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GRAFT VIABILITY AND TRANSFUSION RELATED COMPLICATIONS IN PATIENTS UNDERGOING STEM CELL TRANSPLANTATION

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Graft viability and transfusion related complications in patients undergoing stem cell transplantation

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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ABSTRACT

Allogeneic hematopoietic stem cell transplantation (HSCT) is a treatment strategy for patients with hematopoietic malignancies and inborn errors of metabolism or immunodeficiencies. A successful clinical outcome depends on many factors, such as underlying disease, the patients' status, treatment protocol, donor, graft source and occurrence and severity of complications such as graft versus host disease (GVHD) and infections. The scope of this thesis is to achieve greater understanding of clinical effects and immunological mechanisms of blood group differences and cellular transfusion in patients undergoing HSCT. In addition we investigate the impact of cell graft quality.

HSCT can be performed across the ABO blood group barrier but the impact of blood group incompatibility in HSCT is debated. In **scientific paper I** we analyzed the impact of blood group differences on graft failure (GF). This is a retrospective single center study including 224 patients who underwent myeloablative allogeneic HSCT with grafts from an unrelated donor in 1997-2003. Graft failure (GF) was seen in 6 patients (2.7%). Major ABO mismatch and HLA allele mismatch was significantly associated with GF in the multivariate analysis.

In **scientific paper II** we retrospectively analyzed 310 patients receiving reduced intensity conditioning (RIC) HSCT in 1998-2011. We found no influence of ABO mismatch on overall clinical outcome. However, patients with an ABO mismatched graft required more blood transfusions. We then investigated antibody related complications post-HSCT. Autoimmune hemolytic anemia did not affect overall survival (OS) or transplant related mortality (TRM). Patients with ABO related antibody complications post-HSCT had inferior OS and more TRM. These studies imply that the role of ABO mismatches is not obvious. However, other factors of greater impact may override the effect of ABO donor-recipient differences thus obfuscating its influence.

In **scientific paper III** we retrospectively investigated the impact of HSCT grafts with inferior quality on clinical outcome in 144 patients receiving peripheral blood stem cell grafts. Graft quality was measured as viability of a frozen/thawed control sample. Patients who received grafts with inferior quality developed acute GVHD more frequently and had higher TRM. Grafts with white blood cell count >300 $x10^9$/L had lower viability. In conclusion, graft quality influence clinical outcome after HSCT, hence, conditions for graft storage and handling need to be optimized.

In patients that develop mucositis or breakthrough infections after HSCT, granulocyte transfusions (GCX) can be used. **Scientific paper IV** addresses GCX treatment in 85 patients between 1998 and 2014. GCX can be obtained from donors pretreated with steroids only (S-GCX) or steroids and G-CSF (GCSF-GCX). The overall response to GCX treatment was similar between S-GCX and GCSF-GCX but more complete responses were observed in the GCSF-GCX group. Patients who received GCX due to mucositis benefitted most from GCX whereas the effects of GCX in patients treated due to infection was not as clear. Adverse events (AE) were reported in 36 cases of which 6 were life-threatening or fatal pulmonary AEs. All severe AEs reported were seen in patients treated due to severe infection, further complicating the decision to use GCX treatment in these patients.

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CONTENTS

LIST OF ABBREVIATIONS

AE	Adverse event
AIHA	Autoimmune hemolytic anemia
ANC	Absolute neutrophile count
APC	Antigen presenting cell
ATG	Anti-thymocyte globuline
BM	Bone marrow
CD	Cluster of differentiation
CFU	Colony forming units
CMV	Cytomegalovirus
DAMP	Damage associated molecular pattern
DC	Dendritic cell
DLI	Donor lymphocyte infusion
EBV	Epstein-Barr virus
G-CSF	Granulocyte colony-stimulating factor
GVHD	Graft versus host disease
GVL	Graft versus leukemia
HCT	Hematocrit
HLA	Human leukocyte antigen
HSC	Hematopoietic stem cells
HSC	Hematopoietic stem cells
HSCT	Hematopoietic stem cell transplantation
Ig	Immunoglobuline
IL	Interleukin
LPS	Lipopolysaccharide
MAC	Myeloablative conditioning
MBL	Mannose binding lectin

MHC	Major histocompatibility antigen
NK cell	Natural killer cell
Ns	Not significant
OR	Oddsratio
OS	Overall survival
PAMP	Pathogen associated molecular pattern
PBSC	Peripheral blood stem cells
PLS	Passenger lymphocyte syndrome
PLT	Platelets
PRABO	Persistent or recurring recipient type ABO antibodies
PRP	Pattern recognition receptor
PTLD	Post-transplant lymphoproliferative disorder
RBC	Red blood cells
RIC	Reduced intensity conditioning
RIC	Reduced intensity conditioning
RNA	Ribonucleic acid
SNP	Single nucleitid polymorphism
TCR	T-cell receptor
TH	T-helper cell
TLR	Toll-like receptor
TNC	Total nuclear cells
TPE	Therapeutic plasma exchange
TRM	Transplant related mortality
UC	Umbilical cord blood cells
VZV	Varicella-zoster virus
WBC	White blood cells

1 INTRODUCTION

1.1 THE IMMUNE SYSTEM

The immune system is the body's protection against damaging agents such as pathogens (microbes) causing infection or cancer cells. The immune system can be divided into two major parts, innate immunity and adaptive immunity. Innate immunity is quick to react and consists of barrier protection and cells recognizing conserved structures such as common surface structures of microbes. The adaptive immunity initially reacts slower when encountering a new pathogen but can induce memory for a fast reaction when the pathogen is encountered upon in the future. While the innate system recognizes only a limited number of structures unique to microbes, the adaptive system is able to recognize a much greater number of different targets and not just from microbes. The basis of a functional immune response is its ability to recognize "self" as opposed to "non-self". An immune system reacting towards and killing normal, live cells of a person causes autoimmunity which can lead to disease.

In the context of transfusion medicine and transplantation, knowledge of how the immune system functions is crucial since the recipient (the patient) receiving blood, an organ or stem cells may recognize foreign structures on transplanted/transfused cells as non-self and elicit an immune response.

1.1.1 Innate immunity

The innate immunity, also called natural or native immunity, requires no pre-exposure to a pathogen and can respond quickly. It consists of cellular and biochemical defense mechanisms that are in place before the pathogen is encountered and acts as a first line of defense. The innate immunity consists of several parts; barrier defense, phagocytes, proteins of the complement system, antimicrobial peptides, inflammation and fever.

The physical barrier defense includes epithelial cells on the skin, in the gastrointestinal tract and in the respiratory system. This constant and effective barrier keeps pathogens on the outside. If broken, then the body is exposed to microbes surrounding us. Added to this barrier function some mucosal cells can produce anti-microbial peptides secreted in to the lumen (1).

Inflammation is a process where white blood cells and plasma proteins are recruited from blood, accumulated in the tissue and activated. This process is mediated by pro-inflammatory cytokines produced by cells of the innate immune system. These cytokines, together with components of the complement system, cause blood vessels to dilate and increase permeability inducing the classic symptoms of inflammation: rodor, calor, tumor, dolor and functio laesa (i.e. redness, temperature increase, swelling, pain and loss of function).

Phagocytes are cells that ingest and destroy pathogens and damaged tissue (2). Neutrophil granulocytes and macrophages are the two main types of phagocytes.

Neutrophil granulocytes are polynuclear cells and are the most abundant white blood cell population in the blood circulation. They are relatively short lived (a few days) and an adult approximately produces 10^{11} neutrophils per day. In the cytoplasm of neutrophils there are granules containing enzymes involved in dismantling ingested material (microbes or debris from dead cells) and anti-microbial substances as defensins and cathelicidins. The neutrophil produces reactive oxygen species (ROS), such as hydrogen peroxide, used in killing of microbes (2, 3). It has now been shown that neutrophils also have a role in modulating adaptive immune responses through interactions with T- and B-lymphocytes (4).

Macrophages are either tissue resident cells widely distributed in connective tissue organs or derived from circulating monocytes. The tissue resident macrophages are long lived cells that originally derive from precursor cells in the yolk sac and fetal liver during fetal life. They have differentiated into specialized macrophages displaying different phenotypes, depending on which tissue they reside in. Circulating monocytes derive from committed precursors in the bone marrow and migrate into tissue during inflammation. In the tissue, monocytes mature and differentiate into macrophages (3).

The cells of the innate immune system express pattern recognition receptors (PRP) detecting conserved pathogen associated molecular patterns (PAMPs). PAMPs can be cell-wall components (such as lipopolysaccharide (LPS)) or nucleic acids (such as double stranded RNA) and are unique to microbes. Detection of PAMPs by phagocytes signals presence of microbes and leads to activation of the innate immune response and induction of inflammation. There are several kinds of PRP, the best known are the Toll-like receptors (TLR), a group of transmembrane receptors recognizing for example LPS (5). Macrophages that are activated via TLRs produce pro-inflammatory cytokines (tumor-necrosis factor (TNF), interleukin-1β (IL-1β) and IL-6) that orchestrates inflammatory responses and recruitment of white blood cells. Other examples of PRPs include dectin 1, RIG-like receptors and Nod like receptors (1, 6, 7).

Elements of the immune system can also identify and react to endogenous signals released or expressed by stressed, damaged or dying cells. These signals are called damage associated molecular patterns (DAMPs) (8).

Macrophages can engulf and kill microbes but they also possess the ability to present antigens to and activate T-lymphocytes. Macrophages are so called antigen presenting cells (APCs) and make up an important link between the innate and adaptive immune system.

Another important professional APC is the dendritic cell (DC). Most DCs derive from the same bone marrow precursor as monocytes but some tissue resident DCs, such as the Langerhans cells in the skin, may develop from embryonic tissue. DCs can be divided into different types. Classical DCs resides in an immature form in tissues. When the DCs encounter a pathogen it becomes activated and migrate to the draining lymph node where it presents the microbial antigen to T-cells. Plasmocytoid DCs respond to viral nucleic acids and produce interferons. Follicular DCs resides in the follicles of the lymph nodes and

present antigens recognized by B-cells. In inflammation additional DCs can be recruited. Among the antigen presenting cells (macrophages, B-cells and DCs) the DCs are the most effective.

Dendritic cells express PRPs. Activation of DCs by PAMPs and DAMPs enhances the ability of DCs to process and present antigens to cells within the adaptive immune response (T-cells) and lead to induction of cytokines and expression of additional co-stimulatory ligands needed in T-cell differentiation and expansion. Depending on the nature of the pathogen, the DC will direct the naïve T-cell in to the type of effector T-cell needed.

Another strategy for identification is missing-self recognition. Natural killer (NK) cells, a cell specialized in intracellular pathogen defense; carry both activating and inhibitory receptors. The inhibitory receptors (killer cell Ig-like receptor (KIR)) recognize major histocompatibility complex (MHC) class I, which is normally expressed by all nucleated cells. Many intracellular pathogens, such as viruses, or cell stress causes loss of MHC class I expression on cells. The NK cell can recognize absence of MHC class I on a cell and subsequently kill it. The balance between stimulation of activating and inhibitory receptors is believed to regulate NK cell activation (6, 9, 10). NK cells can respond to stimulation by TLRs as well as more specific antigen recognition receptors. Some KIRs act as activators as do FcγRIIIA (CD16).

1.1.1.1 The complement system

The complement system is a group of proteins reacting as a cascade when activated (11-13). Activation of complement affects the innate immunity resulting in enhancement of inflammation and the adaptive immune response. There are three ways of activating the complement system, all somewhat overlapping and with a common terminal pathway.

Activation by the classical pathway starts with C1q recognizing and binding to pathogens or cell surfaces. C1q recognizes DAMPs, immunoglobulin complexes bound to the surfaces and structures exposed by apoptotic or damaged cells such as phosphatidylserine. The lecitine (or MBL) pathway recognizes mannose containing sugars on pathogens. The third pathway, called the alternative pathway, is constantly activated at a low level and activation is accelerated ("tick-over") when encountering pathogens.

All three pathways can also be activated on the surface of apoptotic cells but are in this context regulated to not activate other functions of the innate immune system in order to protect surrounding cells. This is in contrast to activation by pathogens when the full-blown capacity of the complement system is released.

The common terminal pathway of the complement system begins with cleavage of inactive C3 into C3a (a mediator of inflammation) and C3b (an opsonin). C3b binds to any surface close by leading to opsonization, which promotes phagocytosis, and to the formation of the C5-C9 membrane attack complex causing lysis.

Healthy cells are protected against attack of the complement system by membrane bound structures like membrane bound cofactor protein (MCP or CD46) and complement receptor 1 (CR1 or CD35) or regulators recruited from plasma such as factor H. The protective proteins act as cofactors participating in reactions leading to inactivation of C3b to iC3b or by preventing the formation of C3-convertases (C3 convertases cleaves C3 into C3a and C3b). Cells or pathogens lacking these protective surface regulators cannot control deposition of C3b allowing further complement activation and will, in the end, be eliminated.

1.1.1.2 Cytokines

Within the immune system, cells communicate either by direct contact between receptors and ligands or via extra cellular chemical substances. These substances are called cytokines and are secreted in the extra cellular compartment or in the blood. They consist of proteins and glycoproteins that can regulate and coordinate cell activities. (3, 14, 15). Cytokines are essential for communication between cells of both the innate and adaptive immunity and all cells of the immune system secrete at least one cytokine and often express several cytokine receptors. The nomenclature of cytokines is inconsistent, some are named after the function displayed when discovered, as tumor necrosis factor (TNF) or interferon, and some are called interleukin (IL) along with a number.

Some cytokines act pro-inflammatory (TNF, IL-1β, IL-6) while others, as IL-10, suppress inflammation and immune response. Secreted cytokines can act on cells close by (paracrine signaling) or enter the circulation and function on distant sites (endocrine signaling). Cytokines can also function by autocrine signaling, where the cell is stimulated by its own secreted cytokines, for example IL-2 secretion in activated T-cells.

A large group of cytokines are chemokines (chemoattractant cytokine). There are over 50 different chemokines known and their main function is attracting leukocytes. In general, the CXC chemokines attract neutrophils to a site of inflammation, CC chemokines attract monocytes while both CC and CXC attracts lymphocytes, directing and organizing them in the lymphnode.

In response to pathogens or cell damage, cells of the innate system (macrophages and DCs) secrete cytokines that attracts other immune cells. TNF and IL-1 produced by pathogen-stimulated macrophages stimulate endothelia to produce selectins and integrin ligands (adhesion molecules recognized by neutrophils) and chemokines. This facilitates recruitment, adhesion and subsequent migration of neutrophils, monocytes and T-cells from the blood into the injured tissue.

Some cytokines act in an endocrine fashion when secreted into the blood. IL-6 and IL-1 secreted by phagocytes stimulates synthesis of acute phase proteins in the liver. The acute phase proteins (such as C-reactive protein (CRP)) bind to bacteria and fungi leading to activation of the complement system via C1q and the classical pathway. The blood levels of CRP are elevated during acute inflammatory reactions.

TNF and IL-1 can also act in the hypothalamus to increase prostaglandin production and thereby inducing fever, an elevation of body temperature. The function of fever is not fully understood but it may enhance metabolic activity in immune cells, impair metabolic actions in pathogens and induce behavioral changes in the host thus preventing further injury and infection (3, 15).

1.1.2 Adaptive immunity

The adaptive immunity (also called specific or acquired immunity) recognizes a large number of different antigens with very high affinity (diversity and specificity) and is able to respond more rapidly and vigorously to repeated exposures of the same antigen (memory). Cells of the adaptive immunity are called lymphocytes. There are two types of adaptive immune responses; cellular immunity mediated by T-lymphocytes and humoral immunity where antibodies secreted from B-lymphocytes are the main actors. The mature lymphocytes of the adaptive immunity first exist in a naïve form. Upon activation by an antigen they undergo maturation and clonal expansion. During this process a large number of effector cells are produced. Memory cells are also formed; long lived cells that can react fast when the antigen is encountered the next time. When the pathogen has been cleared, the effector cells undergo apoptosis while the memory cells live on and the immune system returns to its resting state, homeostasis (3).

1.1.2.1 Cells of the adaptive immunity

All lymphocytes originate from bone marrow precursors but then differentiate into T-lymphocytes (T-cells) and B-lymphocytes (B-cells) in response to cytokines (such as IL-7 for T-cells) produced by stromal cells in the bone marrow or thymus. Pro-B- or T-cells early in the maturation process do not express their specific receptors for antigen recognition, T-cell receptor (TCR) on T-cells and immunoglobulin (Ig) on B-cells. They will now undergo a stepwise, complex maturation process starting with genetic rearrangements of their specific antigen receptors. The genetic rearrangements entail production of a very large number of receptor variants using only a relatively small fraction of the genome.

1.1.2.2 T-lymphocytes

The pro-T-cells migrate from the bone marrow to the thymus for further maturation. In the thymus receptor gene rearrangements coding for the T-cell receptor takes place. In T-cells this is referred to as VDJ recombination where one random part from each of the V-, D- and J gene sections are combined to form a unique exon coding for the TCR of the individual T-cell. Genetic differences in the junctions between parts from these segments also add to the genetic diversity (3).

The TCR of T- cells is a heterodimer and consists of one α- and one β-chain (16). There is also a small subset of lymphocytes (<5%) expressing a TCR with one γ- and one δ-chain. These γδT-cells are not MHC-restricted and do not primarily recognize MHC-bound peptides but react rather in a more innate fashion (17).

When the immature T-cell has acquired a functional β-TCR chain they start to express the pre-TCR. The pre-TCR signaling is essential for further T-cell maturation. The T-cell now acquires CD4 and CD8 expression and the function of the TCR is tested through the positive/negative selection process. T-cells that recognize major histocompatibility complex (MHC) class I or II together with a self-peptide and bind with a moderate avidity will be allowed to continue maturation (positive selection). The cell then lose the double CD4/CD8 positivity and commit to one or the other dependent on whether the TCR recognizes MHC class I or II (CD4+ or CD8+, single positive). T-cells that do not recognize either MHC class die by neglect. If a T-cell instead binds MHC with too high avidity the cell is considered potentially harmful and undergoes apoptosis or, in some cases, differentiates into T-regulatory cells. This is called negative selection and protects the body from self-reacting T-cells, i.e. induction of self-tolerance. The T-cells are now mature naïve T-cells and leave the thymus. Naïve T-cells move in the blood and lymphatic system homing to lymph nodes where they can encounter their antigen. If a naïve T-cells do not encounter its antigen, it leaves the lymph node through the lymphatic system into the blood, where it homes to another lymphnode (lymphocyte recirculation).

Within the family of T-cells there are populations with functional differences, the cytotoxic T-cell (CD8+) and the T-helper cell (CD4+).

The T-helper cells act as coordinators directing the immune response through production of cytokines and co-stimulation of other effector cells. There are different types of T-helper cells, Th1, Th2, Th17 and regulatory T-cells (18). Depending on what kind of pathogen the cells of the innate immune have encountered they will secrete different cytokines skewing the adaptive immune response towards the effector cells needed to overcome the pathogen in question.

In case of intracellular pathogens, e.g. interferon-γ (IFN-γ) and IL-12 will be produced by DCs, macrophages and NK-cells promoting Th1 differentiation. The Th1 cell will produce more IFN-γ thus further promoting differentiation of Th1 cells. The differentiation of Th2 cells are triggered by e.g. IL-4 produced by DCs or mastcells in response to helminthes and allergens. Th2 cells are important in the defense against extracellular pathogens; they induce IgE production and activate eosinophils and mastcells. A third type of T-helper cells is called Th17 cells. Th17 cells respond to TGF-β, IL-6 and IL-21 and their primary function is defense against certain extracellular bacteria and fungi. Th17 cells can also induce a broad immunological response and may be involved in tissue inflammation and autoimmunity (19, 20).

Some T-helper cells are regulatory T-cells that inhibit immune responses by production of TGF-β and IL-10, and play an important role for maintenance of self-tolerance (21, 22) Regulatory T-cells can be distinguished using CD25 and FOXP3, although these markers can also be expressed by other cells types. Secreted TGF-β and IL-2 promote differentiation of regulatory T-cells.

Cytotoxic T-cells (CD8+) are crucial in the defense against intracellular pathogens, but also have a central role in the body's protection against cancer (23).To induce activation of cytotoxic T-cells a second signal is required consisting of co-stimulatory molecules by an APC. This can be augmented by T-helper cells by for example cytokine production (the third signal). Cytotoxic T-cells have important effector functions and are after activation able to induce cell death of infected cells. The killing process is highly regulated both in specificity and direction in order to decrease harm to surrounding cells. The cytotoxic T-cell forms a synapse with the infected cell where it releases granule with cytotoxic proteins (granzymes and perforin). Additionally, cytotoxic T-cells express Fas ligand (FasL) that binds to the death receptor Fas which is expressed by many cells, thereby inducing apoptosis.

1.1.2.3 Activation of T-cells

Stimulation and activation of T-cells by their specific antigen is necessary for their survival, further maturation, differentiation and clonal expansion. The TCR binds to antigens in form of peptides presented on major MHC molecules. MHCs are the molecules responsible for direct or indirect activation of the majority of the adaptive immune system. In humans, MHC is also called human leukocyte antigen (HLA). There are two types of MHC, class I and II.

MHC class I is normally expressed on all nucleated cells and display peptides that are synthesized within the cell. The purpose of MCH class I peptide presentation is to report intracellular events. If the cell is infected by an intracellular pathogen peptides from the pathogen will be exposed on MHC class I and can be recognized by cytotoxic T-cells (CD8+) (24, 25).

MHC class II is expressed by APCs; such as DCs and macrophages and B-cells (26) and are recognized by TCRs on T-helper cells (CD4+).

Activation of naïve T-lymphocytes via the TCR requires simultaneous response of co-stimulatory receptors and also presence of specific cytokines produced by cells of the innate immune system. Activation via the TCR without concurrent co-stimulatory activation leads to anergy. In order to induce proliferation and differentiation of the naïve T-cell into effector cells, MHC class II - TCR recognition has to occur in conjunction with co-stimulation via B7 – CD28 and e.g. IL-12 release (27). This leads to release of IL-2 by the T-cell. IL-2 promotes and controls cell differentiation and proliferation in antigen activated T-cells. When the T-cell is activated by an APC, the T-cell produces and up-regulates the IL-2 receptor. IL-2 acts also as an autocrine growth factor and has an anti-apoptotic effect thus promoting cell survival (28).

1.1.2.4 B-lymphocytes

B-lymphocytes (B-cells) belong to the humoral arm of the immune system and their main function is antibody (immunoglobulin; Ig) production. They can also act as a professional APC and express MHC class II (26).Two types immunoglobulins exists, secreted Ig that

neutralize toxins and participate in pathogen elimination and membrane bound Ig that constitutes the B-lymphocyte antigen receptors.

The B-cell develops and matures in the bone marrow. The earliest committed B-cell precursor (pro-B cell) lacks an antigen specific Ig receptor. In the pro-B cell stage the first Ig-gene rearrangements occur, starting with the heavy chains. One D-segment and one J-segment is combined and then joined by a V-segment creating a VDJ-exon. As in T-cells, nucleotide substitutions at the VDJ junctions add to the diversity. The VDJ-exon is kept separate from the Cμ exons by the J-C intron that is retained in the primary RNA script. This intron is removed later by splicing of RNA. The cell can now synthesis the μ heavy chain protein serving as the pre-B cell receptor and has differentiated into a pre-B cell. At this stage the pre-B cell begins rearranging the κ (or λ) light chain by joining one V-segment with a J-segment forming a VJ-exon. The heavy and light chains are assembled into the IgM molecules expressed on the B-cell surface as the B-cell antigen receptor.

If the Ig antigen receptor on the immature B-cell is self-reactive and binds to antigens in the bone marrow with high avidity they will undergo receptor editing or cell death. If the B-cell is not strongly self-reactive it now leaves the bone marrow for the marginal zones of the spleen and lymph nodes where it continues its maturation. A mature naïve B-cell co-express IgM and IgD and re-circulate, i.e. migrate between lymphoid organs where they reside in B-cell follicles. The B-cell can now be activated and respond to an antigen (3).

1.1.2.5 Antibodies and B-cell activation

Antibodies (Ig) consist of chains (two heavy and two light chains) with a variable part in one end that is the antigen binding site and a constant part in the tail end (Fc part). There are five classes or isotypes of immunoglobulin's; IgM, IgG, IgA, IgD and IgE. They differ in the heavy chains and have different characteristics and functions. IgG, IgD and IgE are monomeric whereas IgM is a pentamer and IgA a dimer.

Secreted IgM leads to activation of complement and there by elimination of the pathogen. IgG cause opsonization for phagocytosis by macrophages and neutrophils. IgA is part of the mucosal immunity since IgA is mainly secreted into the lumen of the gut and respiratory tracts. IgE causes mastcell degranulation and thereby hypersensitivity reactions. IgM and IgD are the antigen receptors of naïve B-cells (3, 29-31).

In the context of blood transfusion antibodies of IgM-type towards blood group antigens on red blood cells (RBC) lead to complement activation and subsequent lysis of the RBC within the blood stream (intra-vasal hemolysis). Antibodies of IgG-type commonly opsonize the RBC promoting phagocytosis and thereby removal of the RBC from the blood. However, some RBC antibodies of IgG type are thought to cause complement activation as well and intra-vasal hemolysis (32). The effect mediated by RBC antibodies relies on several factors such as the properties of the heavy chains of the antibody, the antibody concentration, the characteristics of the antigen and the amount of antigen.

When a naïve B-cell encounters a soluble antigen it becomes activated. Depending of the nature of the antigen this can require assistance from T-helper cells. In antibody responses to protein or peptide antigens the antigen is internalized by the B-cell and presented to an activated T-helper cell via MHC class II. The T-helper cell expresses CD40L that bind to CD40 on the B-cell stimulating B-cell differentiation and proliferation. This is referred to as T-dependent antigens.

The B-cell now starts to divide and differentiate. During the antigen-induced proliferation, somatic mutations occur in the genes coding for the variable Ig regions. These mutations gradually lead to higher antigen affinity and is referred to as affinity maturation. A selection process takes place promoting B-cells with the highest affinity. The affinity maturation process is a T-cell dependent, requiring T-helper cells and CD40-CD40L interactions, and takes place in germinal centers of secondary lymphoid organs (i.e. lymph nodes and spleen).

In T-dependent antigen responses the B-cell can undergo heavy chain isotype (class) switching (33, 34). The B-cell changes the constant regions of the heavy chain leaving the variable region (antigen binding part) unaltered and start producing Ig of another class; IgG, IgA or IgE. Class switching is regulated by cytokines produced by the activated T-helper cells. Viruses and intracellular bacteria promote T-helper cells of Th1 type to induce the B-cell switching to IgG1 and IgG3, most likely through IFN-γ production. A Th2 response and IL-4 induces switching to IgE and IgG4. In the gastrointestinal tract TGF-β produced by T-helper cells and other cell types in the mucosa can induce switch to IgA.

In antibody response to non-protein structures (such as polysaccharides, glycolipids and nucleic acids) the antigens are in many cases T-independent, predominantly located in mucosal tissues. B-cells reacting to T-independent antigens typically do not undergo class switch but remain producing IgM and contribute to the production of natural antibodies, i.e antibodies that exists in healthy individuals without apparent antigen exposure (35).

T-independent immune responses generally give rise to short lived plasma cells whereas in a T-dependent response the plasma cells are long lived, residing in the bone marrow continuously secreting antibodies.

In B-cell differentiation memory B-cells are produced capable of generating a rapid response when the antigen is encountered again. The response of naïve B-cells constitutes the primary humoral response and takes days-weeks to accomplish. The secondary humoral response mediated by memory B-cells is much more rapid, Figure 1.

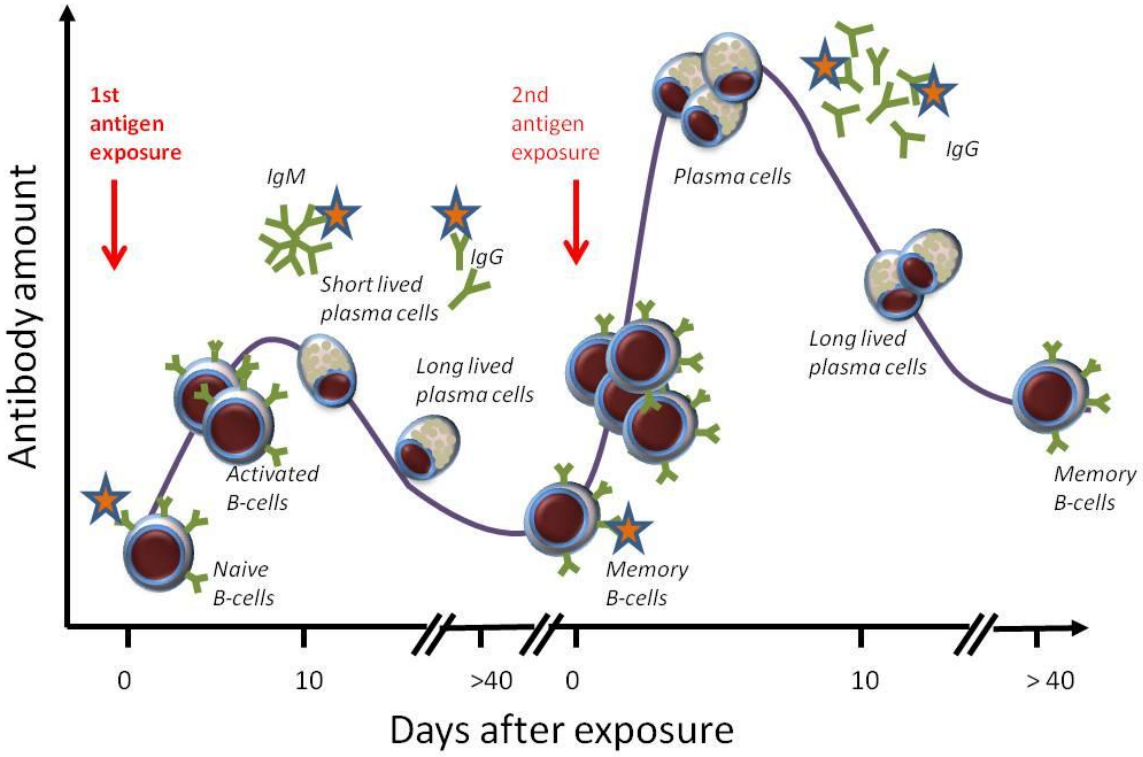

Figure 1. The first exposure to an antigen elicits an antibody response by naïve B-cells. Effector cells and memory cells are produced. In the primary response IgM and subsequently IgG antibodies are produced. The next time an antigen exposure takes place a fast and vigorous B-cell response by memory B-cells is generated, mainly producing large amounts of IgG.

1.2 ALLOGENEIC HEMATOPOIETIC STEM CELL TRANSPLANTATION (HSCT)

Stem cell transplantation can be performed either using autologous stem cells, where the cells serve as a rescue treatment after high dose chemo therapy, or with allogeneic stem cells from a related or unrelated donor. This thesis will focus on allogeneic stem cell transplantation.

In patients with life threatening disease such as hematologic malignancies and inborn errors of metabolism or the immunedeficiencies allogeneic stem cell transplantation is a conceivable curative treatment. The first allogeneic HSCT studies were performed in the late 1950th by E. Donnall Thomas *et al* (36). This was before the discovery of major histocompatibility antigen (MHC), a key feature to success in allogeneic HSCT. In the beginning the results were disappointing and no patient survived the treatment (37). The patients were severely ill patients that died in their leukemia or from graft failure, opportunistic infections and from what would later be recognized as graft versus host disease (GVHD).

With increasing knowledge and experience results have improved vastly. Almost half a century later we now perform over 15 000 allogeneic HSCTs in Europe annually (38).

At our center we perform 80-100 allogeneic HSCTs per year. Survival has improved over the years, in 2006-2009 the overall survival (OS) >3 years was 71% (39).

1.2.1 The HSCT procedure

When a patient is in need of an allogeneic HSCT a search for a suitable donor begins. The patient and his/her siblings are analyzed with regard to their human leukocyte antigen type (HLA), the human version of MHC. If no suitable related donor is available a search for a HLA-matched unrelated donor is performed in the international donor registries. When a potential donor is found the donor goes through a medical examination and if he/she is approved a medical clearance is issued. The patient then begins the conditioning treatment. The hematopoietic stem cells (HSC) from the donor (hereafter called the graft) are collected and transported to the patient (recipient). The graft is analyzed, sometimes processed and then administered to the patient as an infusion. Early after the transplantation the patient is isolated until the leukocytes recover. The patient can be treated in reversed isolation in the HSCT ward or be given conditioning treatment at the hospital followed by a monitored treatment period at home according to the home care program (40-42).

1.2.2 Indications for allogeneic HSCT

The Indications for allogeneic HSCT have varied over time due to emerging new treatments. The main indications today are hematologic malignancies, especially acute myeloid leukemia and acute lymphoid leukemia (2014 EMBT Annual report (www.ebmt.org). Other indications for allogeneic HSCT are lymphoproliferative disorders (Non Hodgkins- and Hodgkins lymphoma, plasma cell disorders), myelodysplastic syndrome (MDS, hemoglobinopathies (as thalassemia and sickle cell disease), bone marrow failure, primary immune deficiencies and inborn errors of metabolism (43).

1.2.3 Human leukocyte antigen (HLA) and transplantation

HLA is the human version of MHC. HLA is inherited and highly diverse, with a wide variety between individuals. The genes coding for HLA are located on chromosome 6. They are divided into HLA class I (HLA-A, HLA-B and HLA-C) and class II (HLA-DR, HLA-DP and HLA-DQ). To determine the HLA type of an individual, genotyping using 6-digit high resolution PCR-SSP for both HLA class I (HLA-A,-B and –C) and II antigens (HLA-DRB1, -DQ1 and -DPA) are performed at Karolinska (44, 45).

1.2.4 Donor selection

To perform an allogeneic HSCT a donor that is HLA-matched on an allele level needs to be identified. According to Mendelian inheritage 25% of siblings could statistically be a matched donor and in 30% of all patients a suitable related donor can indeed be found (46). A sibling donor is the first choice and, if possible, avoiding a female donor to male recipient (43, 47). For the remaining two thirds of the patients a donor may be found through the international donor registries. Today over 26 million donors are registered at the Bone Marrow Donors Worldwide (www.bmdw.org).

A full HLA match (10/10) is desirable when searching for a donor (48) but some HLA-mismatches may be accepted for certain patients on an individual basis (49). When choosing a donor for transplantation additional factors have to be considered, such as cytomegalovirus (CMV) status, sex, age of both donor and recipient and sometimes ABO-blood group. If possible, the best features would be a young male donor who is matched for CMV-status and ABO-type (43, 46). The donor needs to be eligible, i.e. being healthy, tested negative for HIV, HBV, HCV and syphilis and fulfill the requirements stated in EU directives and national legislation.

1.2.5 Conditioning regimes and immunosuppression

Prior to the transplantation the patient receives a conditioning treatment with cytotoxic drugs and or total body irradiation. The purpose of the conditioning treatment is to create space for the new marrow cells, eliminate malignant cells and to prevent graft rejection. The conditioning protocol can be myeloablative (MAC), constructed to eradicate the recipient bone marrow, or reduced intensity conditioning (RIC). RIC protocols cause less organ toxicity and thereby less morbidity (50) and can lower the risk of transplant related mortality (TRM) (51). There are different RIC protocols with varying myeloablative effects, some are regarded as non-myeloablative. The RIC protocols rely on the graft versus leukemia (GVL) effect rather than on the chemo- and/or radiation therapies of the conditioning (52, 53). In RIC there is a prolonged period where donor and recipient lymphocytes co-exists with two different antibody-producing immune systems (54).

The choice of conditioning regimen for a patient is based on protocols determined by disease requirements and the patient's clinical status. In patients with a malignant disease who receive graft from an unrelated donor or umbilical cord blood anti-thymocyte globuline

(ATG) treatment may be added to the conditioning regimen to reduce risk of rejection and prevent GVHD (39, 55).

To prevent GVHD after HSCT immunosuppressive treatment is given. Calcineurin inhibitors, such as cyclosporine A or tacrolimus, in combination with a short course of methotrexate, a drug that suppresses several cell types within the immune system, are the most common regimes (56). The calcineurin inhibitor treatment inhibits T-lymphocyte function and is continued until immune tolerance is achieved; usually 3-6 months post HSCT, provided that the patient does not show signs of GVHD.

1.2.6 Engraftment and graft failure

After HSCT the leukocytes from the donor graft recover in the patient. This is called engraftment. Time to engraftment is defined as the first of three consecutive days when an absolute neutrophil count (ANC) in the patients' peripheral blood reaches $\geq 0.5 \times 10^9$/L and for platelets (PLT) engraftment the PLT count is to be $\geq 50 \times 10^9$/L without platelet transfusions.

Primary graft failure (GF) or rejection is defined as bone marrow hypoplasia (<10% cellularity) with a peripheral ANC $< 0.5 \times 10^9$/L persisting beyond day 21 post-HSCT as confirmed by chimerism analysis with more >95% recipient cells. Patients are considered to have secondary GF if they initially show signs of engraftment and later develop bone marrow hypoplasia requiring frequent transfusions beyond day 60 post HSCT and no signs of donor cells can be detected by chimerism analysis.

1.2.7 Chimerism analysis

In order to assess the graft function in patients post HSCT the fraction of donor / recipient origin of white blood cells, chimerism, can be analyzed (43, 57, 58). Chimerism is also used to diagnose early relapse in patients with malignant disease when a reliable disease specific marker is not available (59). Signs of recipient type cells (of the same cell lineage as the disease) reemerging post-HSCT can be an early sign of relapse. The Chimerism analysis provides important information enabling early therapeutically interventions and thereby better outcome for the patient.

Chimerism analysis of white blood cells is performed on peripheral blood or bone marrow aspirates. Samples are collected from the recipient and donor prior to transplantation and then from the recipient at day+14 after transplantation and onwards according to protocol. After enrichment with immunomagnetic beads (Dynal®), PCR analysis of variable numbers of tandem repeats is used to distinguish donor cells from recipient for T-lymphocytes (CD3), B-lymphocytes (CD19) and myeloid cells (CD33) (60). After 2005 a real-time PCR based on single nucleotide polymorphisms (SNPs) is used for chimerism (61).

Chimerism in the red blood cell population can be assessed by using differences in blood groups between donor and recipient. Prior to HSCT, RBC typing of the donor and the

recipient is performed defining a marker, a difference in blood group between donor and recipient. After HSCT this marker can be used to estimate the proportion of donor- or recipient type red blood cells in the recipient's blood. This was performed at Karolinska on a routine basis until the PCR chimerism of white blood cells was introduced. Today this is performed on request only.

1.2.8 Complications after allogeneic HSCT

The risk of complications after allogeneic HSCT depends largely on the patient's immunological status at a particular time point after HSCT. The main complications after HSCT are infections, GVHD, relapse of the underlying disease and graft failure/rejection.

The rate of the immunological reconstitution after HSCT is slow and dependent on several factors including age, GVHD, conditioning regimen, graft source, donor etc (62). For different cell types this period varies considerably (62-64), thus making the patient susceptible to different infectious agents at different times during the post-HSCT period (65) as illustrated in Figure 2. Additionally, even though cell numbers is restored cell function can be impaired for a considerably longer period.

1.2.8.1 Infectious complications

Barrier defense is a major part of the innate immunity. During allogeneic HSCT the barriers such as the gut mucosa and skin are disrupted by toxic effects of the conditioning regimen. This blazes a trail for bacterial and fungal infections, microbes that normally accommodate on the skin and in the gastrointestinal tract, to become invasive and cause disease. The conditioning regimen also often leaves the patient aplastic until the neutrophils recover after 14-28 days post HSCT (39), i.e. until engraftment. Neutrophils and monocytes are the first cells to recover, closely followed by the NK cells.

Consequently, during the first month after HSCT the patient is very susceptible to infections (66). Both Gram-positive and Gram-negative bacteria, from the skin, mouth and gut pose a problem, as do Candida. For this reason prophylaxis against Candida and bacteria is often given to these patients (66).

The adaptive immunity, T- and B-cells, is in many cases incomplete for a several years. The absolute number of T-cells regenerates quite rapidly within the first months after HSCT. However, the T-cell repertoire and function is still impaired for a long time. Early after HSCT memory and effector T-cells derive from mature T-cells originally present in the graft. Thus, the repertoire of antigen specificity of these T-cells are limited to antigens the donor have encountered prior to graft donation. Hence, the quality of the graft is of vital importance. Immunity against new antigens post HSCT depends on thymic output and the production of de-novo T-cells from hematopoiesis post-HSCT. It has been shown that thymic function, measured as T-cell receptor excision circles (TREC) containing T-cells, deteriorates with increasing age. This can further be influenced by other factors such as graft source (use of PBSC), use of ATG, age and GVHD which all are correlated to decreased TREC levels (67).

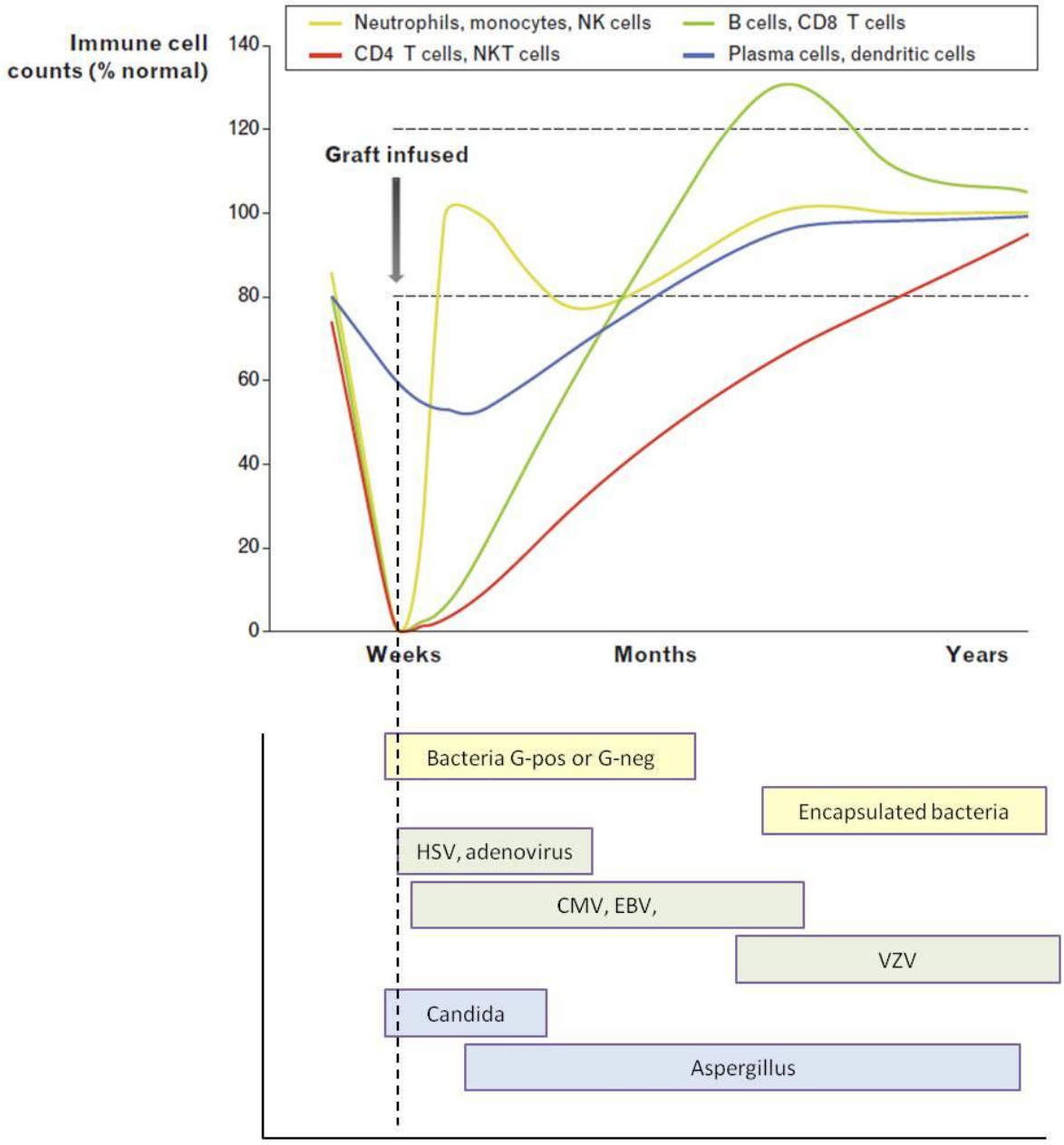

Figure 2: Immune reconstitution of different cell types expressed as cell counts (percentage of normal cell counts) after HSCT. The lower part of the figure shows examples of infections HSCT patients can contract at different time points after HSCT. Adapted from Bosch *et al* 2012 Curr Opin Hematol (63).

Cytotoxic T-cells (CD8+) recover faster than T-helper cells (CD4+). CD4+ T-cells can be of low levels and have incomplete function for years which also affect B-cell function. The B-cells are commonly not detectable the first months post-HSCT and those that can be seen are usually of recipient origin. Recipient type plasma cells have proven to be relatively resistant to conditioning treatment and can subside for months (54).

Subsequently, during the period between engraftment and the first six months after allogeneic HSCT the patients are susceptible to infections normally cleared by cytotoxic T-cells and NK cells, i.e. viral and fungal infections.

After allogeneic HSCT the most common viral infections are caused by herpes viruses; cytomegalovirus (CMV), Epstein-Barr virus (EBV), herpes simplex virus (HSV) and varicella-zoster virus (VZV). In the general population a large proportion is carriers of these viruses. The virus becomes latent after the primary infection and is normally carried through life without causing symptoms.

Immunosuppressed individuals can reactivate CMV and the virus can cause disease in several organs (68). The most important factor for controlling CMV is the T-cell mediated immune response. In CMV infected individuals it is not uncommon to observe a solid cytotoxic T-cell (CD8+) response is seen. In allogeneic HSCT patients lack of T-helper cells (CD4+) is correlated to late CMV infections. The frequency of NK cells increase in CVM infected patients and studies have shown a protective role of certain NK-cell subpopulations against CMV reactivation and infection (10, 69). Matching of CMV-negative donors to CMV-negative patients reduces the risk of CMV infection.

In 90% of the population EBV is a latent infection in the B-cells. In healthy individuals the EBV infection is regulated by specific T-cells but in immune compromised patients control of the infection can be lost resulting in fast proliferation of infected B-lymphocytes causing post-transplant lymphoproliferative disease (PTLD), a condition associated with high mortality. Recipient-donor HLA-mismatch, GVHD and the use of RIC and in vivo T-cell depletion are known risk factors for developing PTLD. PTLD can be treated with the monoclonal anti-CD20 antibody rituximab, donor lymphocyte infusion (DLI) and/or adoptive cellular transfer with EBV specific cytotoxic T-cells from the original donor or from a third party, haplo-identical donor (70-72)

Other seasonal epidemic viral infections common in the community such as adenovirus, respiratory syncytial virus (RSV), calici virus, influenza A and B can cause severe disease and even mortality in the Immunosuppressed HSCT patients (73). Invasive mold infection, especially aspergillus, is a complication with substantial morbidity and mortality after HSCT (74). Air born mold spores exist in the environment, hence total prevention for contracting infection is difficult. Studies have been made to identify patients at risk allowing for emptive or early treatment (74-76) .

Viral infections and encapsulated bacteria such as *S. pneumonia* and *H. influenza* are still a potential problem in the later phase after HSCT (>100 days) especially if the patient develops chronic GVHD. Prophylaxis against *P jirovecii* is mandatory after HSCT.

1.2.8.2 Graft versus host disease (GVHD) and graft versus leukemia (GVL)

When the two immune systems of the recipient and the donor meet after HSCT reactions in both directions can occur, either rejection where the recipients (hosts) immune system reacts against the graft, or in the other direction where the immune competent cells from the graft reacts against the recipients cells (graft-versus-host). This is alloreactivity; immunological reactions occurring after transplantation between individuals of the same species.

Graft versus host disease (GVHD) is one of the main challenges associated with allogeneic HSCT and its severe forms are related to high morbidity and mortality (43). GVHD is divided into an acute and a chronic form. At our center approximately 40% of the HSCT patients develop acute GVHD grades II-IV and 30% develop chronic GVHD (39).

Acute GVHD typically arises within the first 90 days after HSCT and mainly affects the skin, gut and liver. Acute GVHD is graded from I to IV. The classical description of the underlying pathophysiology involves an initiation phase with tissue damage and/or pathogens (expressing lipopolysaccharide; LPS). This tissue damage is promoted by the conditioning treatment and activates host antigen presenting cells (APC). The host APC then presents antigens to donor T-lymphocytes. Alloreactivity is induced, as is the release of inflammatory cytokines such as tumor necrosis factor (TNF)-alfa, IL-1 and IL-2, leading to recruitment of other effector cells including neutrophils, NK-cells and macrophages. A cycle of inflammation and tissue injury is induced and maintained (77-79).

Chronic GVHD typically arises later, more than three months after HSCT and resembles more the clinical picture of an autoimmune disease with fibrotic features. It mainly affects skin, lungs, liver, kidney, gut and oral- and eye mucosa but can occur in any organ. Both T- and B-lymphocytes play important roles in the underlying pathophysiology of chronic GVHD but how they interact and their mode of action is still not fully known (78, 80). It has long been suggested that chronic GVHD depends mainly on the skewing of CD4+ T-helper cells towards the Th2 phenotype whereas acute GVHD is predominantly a Th1 process. However, this paradigm is questioned with reports of a mixed Th1/Th17 phenotype in skin from patients with cutaneous chronic GVHD (81, 82).

While alloreactivity by cells of donor origin towards cells of the recipient is referred to as GVHD, the reaction inflicted by donor cells towards malignant cells in the recipient is termed graft versus leukemia (GVL) or graft versus tumor (GVT) effect. In 1990 Horowitz *et al* published a study showing decreased relapse in patients with GVHD reactions and those that had received non-T-cell depleted grafts (83), findings that supported the GVL effect described earlier (84).

Patients with a syngeneic (identical twin) donor have been shown to have increased risk of relapse when compared to patients with HLA-matched sibling and no signs of GVHD (83). This implicates that genetic disparity between donor and recipient has an important role in GVL. The mechanism behind GVL and the distinction between GVL and GVHD has been

studied extensively (85). T-cells play a major role (86-88) and the use of donor lymphocyte infusions (DLI) after HSCT has been shown to mediate GVL effect (52, 89, 90). Other cells such as DCs and NK cells have also been suggested to take part in the mechanism of GVL (78, 91-94).

The discovery of the GVL/GVT effect has had fundamental implications on the treatment modality HSCT per se. The use of reduced intensity conditioning (RIC) protocols relying on the GVL effect rather than on the chemo- and/or radiation therapies of the conditioning (52) have emerged thus permitting transplantation of patients with malignant disease that would not tolerate the more toxic myeloablative protocols (95-97).

In summary, outcome of the HSCT patient rely on that the immune systems of recipient and donor origins to acquire tolerance and to balance the risk of rejection with the occurrence of GVHD (98). In patients with malignant disease harnessing the potential harmful effects of GVHD and yet maintaining sufficient GVL effect is a key feature.

1.3 HSCT GRAFTS

In allogeneic HSCT grafts from different sources are used, commonly peripheral stem cells (PBSC), bone marrow (BM) or umbilical cord blood cells (UC). Which graft source to use for a patient depends on donor preference and HSCT indication (43, 99). PBSC has been shown to give faster engraftment (100). Since it is known to give more GVHD it is preferred in malignant diseases since an increased rate of mild GVHD decreases the risk of relapse (43, 83, 101). Due to this increased risk of GVHD BM is preferred in patients with non-malignant disease. The overall most commonly used graft source today is PBSC (38).

HSCT grafts contain hematopoietic cells in different stages of maturation, from stem cells expressing the CD34 marker on their surface to mature cells found in peripheral blood. Grafts of different sources contain different amounts of cells, Table 1 (102). The different graft sources do not just have different cell content in absolute numbers; the cells in the grafts also possess different characteristics.

1.3.1 Bone marrow (BM)

BM is collected under full anesthesia by repeated aspirations from crista illiaca. The aspirated bone marrow is filtered through a blood transfusion filter (170nm) into a bag. Anticoagulant in form of either ACD-A alone or ACD-A in combination with heparin is added.
BM often contains larger volume, more red blood cells but less white blood cells and hematopoietic stem cells as compared to PBSC (Table 1)(102). The target cell dose for transplantation is at least >2 $x10^8$ TNC/kg recipient body weight (43). Due to the large amounts of red blood cells in BM, ABO mismatch between donor and recipient have to be considered. In major ABO mismatches the BM may need processing before transplanted. Stimulation of bone marrow donors with G-CSF have been tried to achieve a larger cell dose, thus speeding up engraftment (103)

Table 1		PBSC	BM	UC
Volume	mL	364 (218-1672)	945 (218-1672)	26 (18-212)
White blood cells	$x10^9$ /L	212 (156-368)	48 (8-154)	37 (15-114)
Red blood cells (HCT)	%	1.4 (0.8-2.3)	32 (20-41)	-
Platelets	$x10^9$ /L	1275 (240-3640)	110 (32-242)	-
CD34+ stem cells	$x10^6$ /L	875 (162-3760)	164 (18-918)	91 (8-654)
T-lymphocytes (CD3)	$x10^9$ /L	322 (23-3760)	2,3 (1-10)	-
B-lymphocytes (CD19)	$x10^9$ /L	10 (2-28)	0.3 (0.1-2.7)	-
NK cells (CD56/16)	$x10^9$ /L	5 (3-19)	0.2 (0.1-0.7)	-
TNC /kg body weight	$x10^8$ /kg	11 (4-18)	4.0 (1.0-13)	0.34 (0.16-1.6)
CD34+/kg body weight	$x10^6$ /kg	3.0 (0.3-10)	4.4 (0.9-12.6)	0.1 (0.02-0.6)

Table 1: Contents in allogeneic HSC grafts after collection from peripheral blood stem cell (PBSC; n=52) or bone marrow (BM; n=44) grafts at Karolinska 2013-2014. PBSC grafts are all collected at Karolinska from related donors, most collections are performed on Spectra Optia. BM grafts are from pediatric donors, related and unrelated adult donors and collected at different centers. Umbilical cord blood (UC) units transplanted at Karolinska 2009 (n=14), the numbers depicted are pre-freeze values obtained from the cord blood banks.
Figures depict median values with range in brackets. (Unpublished data).

1.3.2 Peripheral blood stem cells (PBSC)

When collecting PBSC the donor is stimulated with G-CSF injections during the five days prior to the first collection. The collection is performed using aphaeresis technique, most commonly via needles in peripheral veins. The collection takes 4-6 hours where usually a volume corresponding to three blood volumes are processed. The target cell dose for transplantation is 5-10 $x10^6$ CD34+/kg recipient body weight (43), two collections may be needed to achieve target dose. The PBSC graft differ slightly from BM grafts not just in blood cell numbers but also in cell composition (102, 104), with T-cells skewed towards Th2 cytokine production, promoted expansion of T regulatory cells, induced IL-4 and IL-10 production and impaired cytotoxicity of NK cells (105).

1.3.3 Umbilical cord blood (UC)

UC is most commonly collected on voluntary basis from umbilical cord and placenta after birth. UC can be separated by centrifugation using dextran or HES after collection to reduce volume and deplete contaminating red blood cells (106, 107). However, as with all cell processing this results in cell losses why cryopreservation without prior separation is preferential if cell numbers are crucial. The UCs are cryopreserved and kept by UC banks, usually in nitrogen storage tanks. UC was originally mainly used in pediatric patients due to a small total cell dose and their richness in stem cells. However, UC is an alternative also in adult patients who lacks a suitable related or unrelated donor (61, 108-111). The target cell dose for UC transplantation is >3 x10^7 TNC /kg recipient body weight (43). This can be difficult to achieve in adults hence transplantation using two UC units can be used (double UC) (111).

1.3.4 Graft storage and transportation

In about two thirds of all allogeneic HSCT performed today a suitable HLA-matched related donor cannot be identified (46). In these cases an unrelated HLA-matched donor may be found through the international donor registries. Cell grafts from unrelated donors are almost always collected at distant collection sites with storage and transportation of cell grafts becoming a crucial link in the transplantation process.

The conditions under which HSCT grafts are stored and transported have been studied earlier (112-117). Cellular graft source, cell concentration, temperature and storage/transport time have been described as factors influencing cell quality. Storage temperature has been shown to affect clinical outcome with a lower incidence of graft failure in patients whose grafts were stored at 4 °C compared to room temperature (118).

The maximum storage time of HSC (PBSC in particular) is temperature dependent (112, 113). Jansen *et al* (113) have shown that after 48 hours of storage cell viability decreases rapidly with rising temperature. In a study by Antonenas *et al* (112) it was shown that PBSC grafts lost significantly more viable CD34+ cells when stored at room temperature compared to storage in 4 °C. For BM there was no significant difference between storage temperatures. Allogeneic PBSC grafts were shown to lose significantly more viable CD34+ cells than autologous grafts during storage, especially in room temperature. They speculated that higher WBC and platelet counts in allogeneic PBSC grafts may have caused this faster deterioration.

In Sweden, while allogeneic HSC grafts should be infused as soon as possible, the maximum limit for PBSC kept in 4°C is set to 72 hours.

1.3.5 Analysis of cell quality and viability

Upon arrival of the grafts they are analyzed for number and recovery of CD34+ stem cells and sometimes also their ability to form colony forming units (CFU) (119). However, recovery of CD34+ cells can be difficult to assess due to variation in analysis between laboratories. The viability of nucleated cells in the graft can either be measured by

microscope based assays such as trypan blue-staining or via flow cytometry based assays by staining using 7-Aminoactinomycin D 7AAD or propidium iodine (PI). These methods detect dead cells but not cells in early apoptosis.

All cell products are tested for microbial contamination (120).

1.3.6 Graft processing

HSC graft processing is generally performed for two reasons, either depleting or washing the graft to avoid adverse effect during infusion or enhancing/purging/selecting/depleting parts of the graft to achieve long term effects. In any cell processing the indication for the intervention has to be assessed in relation to the risk of cell-loss in the graft.

1.3.6.1 Avoiding adverse events

In major ABO incompatible HSCT using bone marrow red blood cell (RBC) depletion of the graft is often necessary due to recipient antibodies against donor ABO antigen on RBCs to avoid adverse events during infusion. RBC depletion can be done using different instruments (Cobe 2991, Cobe Spectra, Spectra Optia, Sepax) (121, 122). By centrifugation a buffy coat can be prepared thus depleting plasma and RBCs, and, if additional reduction of RBCs is required, "double buffy coat" can be performed (123). In the "double buffy coat" packed RBCs (from a community blood donor) of blood group O is added to the first buffy coat diluting the remaining incompatible donor RBCs. The graft is then centrifuged again producing a second buffy coat with only few remaining incompatible donor RBC. An alternative for RBC depletion gradient density centrifugation with (e.g. Lymphoprep™) can be used as an alternative to RBC depletion (124).

In minor ABO mismatches plasma can be depleted by centrifugation before infusing grafts to avoid incompatible ABO antibodies in plasma.

Graft volume reduction is sometimes warranted, especially in pediatric recipients (125). This can be achieved by centrifugation and depletion of plasma supernatant or by producing a buffy coat.

1.3.6.2 Graft processing for more long term effect

In cases where a positive selection of a specific cell type is warranted selection using magnetic beads can be performed with for example the CliniMACS system. In pediatric patients with neuroblastoma engaging the bone marrow CD34 positive selection of autologous grafts can be performed to avoid contaminating cancer cells in the graft (126).

Magnetic bead selection can also be applied to deplete unwanted cells. The T- (CD3) and B-cell (CD19) depletion has successfully been used (127) to avoid GVHD in haplo-identical allogeneic HSCT. Today, depletion of αβT-cells can be used for avoiding GVHD in patients undergoing allogeneic HSCT with mismatched donors (128) or as stem cell boost in patients with secondary graft failure (129, 130). The majority of T-cells in peripheral blood (95%)

express the αβT-cell receptor (TCR) where as 5% express the γδTCR. The γδT-cells are not strictly regulated by MHC molecules and are thus less likely to cause HLA-dependent GVHD. The rationale behind sparing the γδT-cells in graft processing is that they have been shown to protect against leukemia relapse (131, 132) and been associated with a protective role against cytomegalovirus (CMV) reactivation and disease (133).

1.3.6.3 Cryopreservation

To preserve cells for future use autologous HSC and allogeneic lymphocytes for donor lymphocyte infusions (DLI) can be cryopreserved. Allogeneic HSC are generally not cryopreserved due to increased risk of graft failure (134).

Cryopreservation methods are described in more detail in the materials and methods section.

1.3.6.4 Adverse events related to cell infusions

At Karolinska 399 hematopoietic cell products were issued during 2014 from our cell processing lab. Adverse events (AE) during or after infusion were reported for 95 of these products (unpublished data). Most were minor AE such as shivering or nausea but severe AE did occur with neurological symptoms, cardiac arrhythmias and anaphylaxis.

DMSO-related AEs in patients during infusion of cryopreserved cells are common (135). Washing the cells reduces the severe AEs but does not totally remove DMSO. AEs attributed to DMSO can still occur, especially allergic reactions (136). Pre-treatment of the patients with steroids and anti-histamine, regardless of thawing method, is recommended.

Examples of other AEs that can occur during or after cell infusion are nausea, vomiting, fever, shivering, rash, erythema, hemoglobinuria, hypo- or hypertension, arrhythmias, tremor or neurological symptoms with convulsions, amnesia or affected consciousness. AEs can be severe, even life threatening or lethal, especially when giving cells that have been cryopreserved (135, 137-139).

1.4 BLOOD GROUPS

In the turn of the last century Karl Landsteiner discovered that serum from some of his research subjects agglutinated red blood cells (RBC) from others in a certain pattern (140). The results were published in 1901 and led to the discovery of the blood groups A, B, O and AB. Others have continued the work of describing human blood groups and their clinical importance (32, 141, 142).

Today, close to 300 blood group antigens have been described and registered at the International Society of Blood Transfusion (ISBT). Blood group antigens can be sorted in to four categories; systems, collections, low-incidence antigens and high-incidence antigens (143). A system can consist of one or more blood group antigen. The antigen must be defined by a human antibody targeting the antigen and must be inheritable. The genes coding for the blood group antigen have to be identified and sequenced, have a known chromosomal location and be unique, i.e. not cross-linked homologues from other genes encoding for other blood group systems (www.isbtweb.org). If an antigen does not fulfill these blood group system criteria it will be categorized as a collection, a low- or a high incidence antigen. The genes coding for blood group antigens are located on different chromosomes throughout the genome. Most blood group allelic differences are single nucleotide polymorphisms (SNP) in exons, introns or regulatory regions. Other genetic variation such as crossover between homologous genes (as RH or MNSs), nucleotide deletions or insertions causing a shift in the reading frame (ABO)or whole or partial gene deletions (RH, MNSs, H) exist (144).

Differences in blood groups between individuals can evoke an immune response after blood transfusion. The recipient of a blood transfusion can form irregular antibodies against blood group antigens present on transfused red blood cells, i.e. alloimmunization (32). Hence, compatibility testing is mandatory prior to transfusion of blood. However, not all patients form irregular antibodies after transfusion and the mechanisms behind this immune response is not fully understood. Studies of the immune response after blood transfusion and identifying patients at risk have been made (145-149).

A blood group is thus an allelic difference of a molecule expressed on the surface of RBCs. These molecules have different functions in the cell, such as transporters, channels, receptors, adhesion molecules, enzymes or structural proteins and many still unknown functions (150-152).

Blood groups, although defined on red blood cells, are expressed on many other cells and tissues in the human body (153, 154) and can also be present in other species and plants (155, 156). The role of blood groups and the genetic differences in these structures between individuals is debated.

During RBC development and differentiation blood groups appear at different time points (157-162). Several studies have investigated blood group antigen expression at different time

points in cell cultures from CD34+ selected cells. This is illustrated in Figure 3. Of the blood group antigens analyzed K2, A and RHAG (Rh50) are among the earliest antigens to be expressed. Notably, ABO is expressed quite early in erythropoiesis.

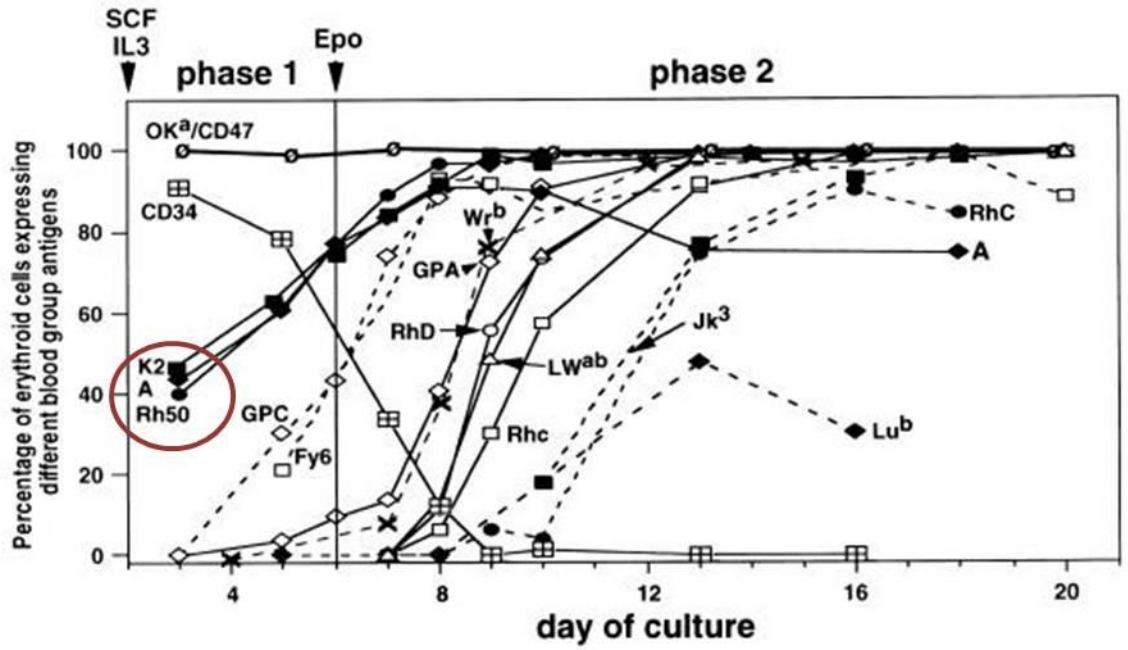

Figure 3: Expression of blood group antigens during erythroid cell differentiation. In this study erythroid cells in cell culture (CD71^{++} or GPA^{+}) were analyzed for blood group antigen expression using human or mouse MAbs or human sera. The results are expressed as the percentage of erythroid cell subset expressing a given antigen (without indication on their quantitative level of expression) at different time points during culture. Expression of A, K2 and RHAG is detected early in the cell cultures (circled). Adapted from Bony V. *et al* Br J of Haem 1999 (158)

1.4.1 ABO

The ABO blood group system is the clinically most important blood group system due to the occurrence of isoagglutinins, antibodies normally present in all persons older than six months of age. These directly agglutinating antibodies, anti-A and anti-B, are of IgM type, reactive at 37 °C and can activate complement *in vivo*. If ABO-incompatible blood is transfused to a person, an instant, intra-vassal hemolysis accompanied by severe symptoms such as fever, shivering, back-pain, anuria, hematuria, disseminated intra-vassal coagulation and circulatory chock can be expected. The reactions can be severe and even lethal (32).

ABO antigens A and B are carbohydrate structures present on many cells and tissues. To the current day around 100 ABO alleles have been described. The genes encode for transferases that put N-acetyl-D-galactoseamine (blood group A) or D-galactose (blood group B) on the precursor chains (H) forming the ABO-active glycoproteins. The A and B transferases differ by only four amino acids (163-165).

1.4.2 ABO and transplantation

Because A and B antigens are distributed throughout the body and anti-A & anti-B are present as preformed ABO-antibodies, the ABO-system plays a pivotal role in transplantation (166), illustrated in Figure 4.

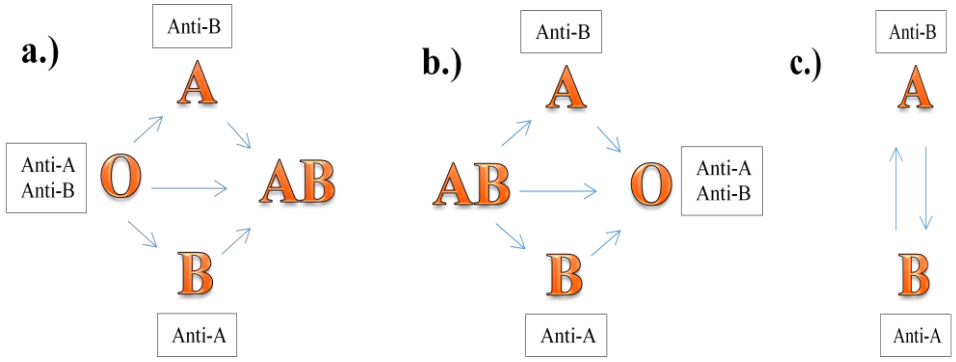

Figure 4: Different types of ABO mismatches that can occur in transplantation:
a.) Minor ABO mismatch where the donor has antibodies against recipient ABO antigens,
b.) Major ABO mismatch where donor antigen is given to a recipient with preformed antibodies,
c.) Bidirectional ABO mismatch where the recipient has antibodies against donor antigens and the donor has antibodies against recipient antigens, i.e. major and minor ABO mismatch.

In liver transplantation, a major ABO mismatch is correlated to an increased risk of rejection and post-transplant complications (167) but a minor ABO mismatch can also affect outcome (168). In kidney transplantation a major ABO mismatch is generally not feasible without pretreatment of the patient to remove the ABO antibodies. Pretreatment protocols combine immune-adsorption aphaeresis with rituximab (anti-CD20) treatment and immunosuppressive drugs to remove ABO-antibodies, thus enabling ABO-incompatible kidney transplantation (169, 170).

ABO antigens are expressed quite early in hematopoiesis, but hematopoietic stem cell transplantation over ABO-barrier can be performed (43, 171, 172). In relation to the HLA-genes, ABO is carried on a different site of the genome and is inherited independently. Hence, a related or unrelated donor can be HLA identical but have different ABO blood group. The effect of ABO on clinical outcome of allogeneic HSCT will be discussed later.

The occurrence of isoagglutinins after transplantation is dependent on the immunological reconstitution after HSCT. Regular antibodies against ABO antigens not present on recipient nor donor cells decline quite rapidly after transplantation and increase again 30 – 50 days post-transplant. In minor ABO mismatched transplants antibodies against recipient ABO antigens are usually not formed. Recipient ABO antibodies against donor antigens in a ABO major or bidirectional setting disappear within the same time period and usually remain undetectable (173, 174). In patients that receive grafts from unrelated donors, the recipient ABO antibodies decline faster than in cases where related donors are used (175).

1.4.3 Minor ABO mismatch and Passenger lymphocyte syndrome

In transplantation of organs or hematopoietic stem cells, mature B-lymphocytes accompany the graft. If the transplantation is a minor ABO mismatch, some of these mature B-lymphocytes may produce antibodies with ABO specificity against antigens on recipient red blood cells. This can give rise to passenger lymphocyte syndrome (PLS), a condition that usually occurs 7-14 (-30) days after transplantation and can cause abrupt, mild to severe hemolysis in the recipient. The antibodies disappear and the condition usually resolves within weeks (176). The treatment is supportive. PLS is common after organ transplantation, particularly after lung and liver transplantation (177).

In HSCT the picture is more complex. Some centers report frequent PLS after HSCT (178, 179), while others rarely see this phenomenon (scientific paper II).

1.4.4 Major ABO mismatch and persisting red cell antibodies

After a major ABO mismatch, recipient (host) antibodies against donor ABO antigens usually disappear after a few weeks (173, 174). It is reported that patients undergoing major ABO incompatible HSCT receive more red blood cell transfusions (180).
However, in some cases the recipient ABO antibodies persists and can do so for up to a year. This causes delayed hemolysis, delayed engraftment of donor red blood cells and pure red cell aplasia (PRCA). The patients become transfusion dependent for a long period of time with accompanying risk of iron overload (181). An increased incidence of transplant-associated microangiopathy (TAM) (182) has also been reported in major ABO mismatched HSCT

1.4.5 Irregular antibodies after transplantation

Development of irregular antibodies after allogeneic HSCT can occur. If the corresponding blood group antigen is present on other cells and tissue, both the recipient and the donor need to lack the antigen in question for the patient to form antibodies.

Irregular antibodies against non-ABO blood group antigens can also cause PLS after transplantation of organs and HSC (183-185).

1.4.6 Autoimmune hemolytic anemia (AIHA)

Autoimmune hemolytic anemia (AIHA) is a known complication after allogeneic HSCT with an incidence of 2% to 6%, affecting both adults and children. The median time of onset of AIHA is between 5 and 12 months after HSCT (186, 187). Here, the donor lymphocytes produce autoantibodies against donor antigen. An AIHA after HSCT is generally more refractory to treatment than AIHA in the general population.

1.5 GRANULOCYTE TRANSFUSION

After allogeneic HSCT temporary severe neutropenia is common (41). In many cases, the tissue damage caused by HSCT conditioning leads to mucositis, resulting in ulceration and inflammation of the mucosa, bringing severe pain, difficulties with enteral nutrition and diarrhea (188, 189). Neutropenia and mucositis result in increased susceptibility to infection due to damaged barrier function and a severe reduction of immune cells (190).

In some patients with severe infection and neutropenia after HSCT or chemotherapy, antimicrobial treatment is not sufficient. In these cases transfusion of granulocytes from relatives or community donors can be a valuable treatment option (191-193). Granulocyte transfusions (GCX) have also been used at our center as a bridge to engraftment in patients with severe mucositis after HSCT.

Granulocytes can also be given as prophylaxis in neutropenic patients at risk of severe infections in allogeneic HSCT (194).

1.5.1 Granulocyte products for transfusions

Granulocytes are most often obtained by leukapheresis. In some countries, pre-treatment of voluntary community donors using G-CSF is not recommended and alternative ways of obtaining granulocytes concentrates have been described using pooled buffy coats from whole blood donations (195, 196).

Prior to transfusion granulocyte products must be irradiated (>25 Gy) to avoid transfusion associated GVHD. When transfusing granulocyte products a 170 µm transfusion aggregate is used. Granulocyte products are transfused within 24 hours after collection, most are given within hours of collection.

It is debated whether the increment of neutrophils in peripheral blood and clinical outcome; survival, infection control and adverse events, is correlated to the number of granulocytes in the product and donor pre-treatment. (191, 197-199).

1.5.2 Granulocyte aphaeresis

During granulocyte collection by leukaphaeresis, sodium citrate is used as anticoagulant. Granulocytes and red blood cells have a similar density. To separate the granulocytes from the red cells, a starch is added as a continuous infusion together with the anticoagulant in the access tubing. Different starch brands have been used over the years (200). Typically in granulocyte collections at our center, 7000 mL of whole blood is processed at a rate of 60-65 mL/min collecting 3mL/min of granulocyte concentrate.

1.5.3 Selection and pre-treatment of granulocyte donors

Granulocyte donors are healthy, eligible and matched for ABO. ABO has to be matched due to the high amount of red blood cells in the granulocyte product. If the patient is CMV-negative then CMV-negative donors are chosen.

Historically, GCX donors were not pre-treated resulting in granulocyte products containing insufficient numbers of cells. Subsequently, pre-treating donors with steroids mobilizing granulocytes from the marginal pool or steroids combined with G-CSF, stimulating granulocyte proliferation, have increased the harvest yield significantly (197, 201, 202). At our center donors are pre-treated with 3 mg oral dexamethasone and 0.3 mg G-CSF (filgrastim, Neupogen, Amgen, Munich, Germany) as a single subcutaneous injection 12 hours prior to collection (203, 204).

1.5.4 Effect of granulocyte transfusions

Clinical effects of GCX treatment have been studied over several decades with inconclusive results (191, 199, 202, 205). Interpretation of results is complicated by the wide variations in study designs, treatment indications, treatment protocols and pre-treatment protocols (191, 192, 202, 206-208). In the Cochrane review by Stanworth *et al* from 2005 (reprinted in 2010) (193) the reviewers concluded that the evidence for GCX treatment in neutropenic patients with infection is inconclusive. A recent Cochrane review by Estcourt *et al* (2015) (194) regarding prophylactic use of GCX in neutropenic patients concluded that there is low-grade evidence of decreased risk of bacterial- and fungal infections and that this effect may be dose-dependent. There is a need for further studies, including comparison of clinical efficacy between granulocytes obtained by different pre-treatment protocols. The prospect of randomizing potentially lifesaving treatment has proven to be difficult (209). Further studies with other approaches (intention to treat) are on the way (210).

2 AIMS

The main objective of this thesis was to achieve better understanding of the impact of blood group differences and transfusion related complications on clinical outcome after allogeneic stem cell transplantation (HSCT). Additionally, the influence of graft quality on clinical outcome was studied.

The specific aims of the present thesis are:

- To evaluate the influence of blood group differences between donor and recipient on clinical outcome in allogeneic HSCT and to study the clinical implications of red blood cell antibodies in these patients.

- To study the effect of graft quality on clinical outcome in allogeneic HSCT using a viability analysis on frozen/thawed samples

- To evaluate the effect of granulocyte transfusions in patients with severe infection or mucositis after allogeneic HSCT and the impact of donor pre-treatment.

3 MATERIALS AND METHODS

3.1 ETHICS

All studies in this thesis work were approved by the regional ethical review board in Stockholm.

3.2 SUBJECTS AND METHODS

3.2.1 Scientific paper I

Scientific paper I is a retrospective study including 224 patients with leukemia who underwent allogeneic HSCT with grafts from unrelated donors between 1997 and 2003 at Karolinska University Hospital. There were 120 males and 104 females. The median age were 29 years (range 1-55). The diagnoses were acute leukemia (n=139), chronic leukemia (n=71) and myelodysplastic syndrome (n=14). All received myeloablative conditioning and anti-thymocyte globuline (ATG). Immunosuppression consisted of cyclosporin A and methotrexate (n=210) or cyclosporine and prednisolone or mycofenolate mofetil (MMF) (n=14). Graft source were bone marrow (n=152) or PBSC (n=72). Two hundred patients had HLA-A, -B, and -DR identical donors and 24 patients had an allele-level mismatched donor (2 HLA-A, 9 HLA-B and 13 HLA-DR).

3.2.2 Scientific paper II

In this retrospective study 424 consecutive patients who underwent HSCT with reduced intercity conditioning (RIC) between January 1998 and August 2011 at our hospital were included. Of these, 114 patients with early malignant relapse (<12 months) or those who underwent re-transplantation were excluded due to short follow-up time, leaving 310 patients for analysis. The median follow-up time was 24 month (range 0.2-151 months) for all patients and 46 months (range 1.4-151) for surviving patients.

Twenty-three patients with malignant disease and 12 with nonmalignant disease suffered from graft failure. Out of these 35 graft failures, seven patients had a sibling donor, 21 had a matched unrelated donor, and 7 had mismatched unrelated donors. Non-myeloablative conditioning was used in 9 patients and RIC in the remaining 26 patients. Graft sources were 5 cord blood, 20 PBSC, and 10 BM.

The patients were divided into 3 groups based on ABO compatibility (ABO match, ABO minor mismatch and ABO major mismatch). Bidirectional ABO mismatches were included in the major ABO major mismatch group. Clinical outcome was analyzed with regards to ABO match between donor and recipient.

We investigated each hemolytic complication that could arise from ABO incompatible transplants; passenger lymphocyte syndrome (PLS), persistent or reoccurring recipient ABO antibodies (PRABO) and also autoimmune hemolytic anemia (AIHA) and their impact on clinical outcome. Data from each patient documented in the Transfusion Medicine Laboratory Information System was retrospectively analyzed for results of direct anti-globulin test DAT and indirect anti-globulin test (IAT), transfusion history, and presence of detected anti-ABO antibodies. The number of transfused RBCs and platelet units during the first 2 and 12 months post-HSCT, respectively, were noted. Patients who fulfilled criteria for PLS, PRABO or AIHA were identified.

We also analyzed transfusion requirements with regards to ABO differences between donor and recipient. Transfusion requirements were measured as number of transfusion (units) of packed red blood cells or platelets given at 0-2 months post HSCT and 0-12 months post HSCT.

3.2.3 Scientific paper III

Our aim in scientific paper III was to evaluate the influence of graft viability on clinical outcome. This was a retrospective, single center study including 144 patients receiving PBSC grafts from unrelated (n=138) or related (n=6) donors. All grafts were collected at other centers. Quality the grafts was assessed by the viability of white blood cells (WBC) on a frozen-thawed sample from the PBSC grafts.

Prior to transplant the PBSC grafts were divided into two parts. The main part was infused into the patient for transplantation. A smaller part was cryopreserved in escalating doses based on the amount of CD3+ cells for future use as donor lymphocyte infusion (DLI). A control vial was frozen together with the DLI doses to allow viability testing after cryopreservation. This is illustrated in Figure 5.

In this study the median viability of the frozen-thawed sample was 64%. To analyze the impact of graft viability on clinical outcome this median value was set as a cutoff, i.e. a viability of less than 64% was considered as inferior graft quality. To compare the two patients groups (those with graft quality less than or more than 64%) univariate analysis was performed with regard to the clinical outcomes such as TRM, OS, relapse, and acute GVHD. Viability was also analyzed as a continuous variable by univariate analysis.

A multivariate analysis was performed on the clinical variables (OS, aGVHD, TRM, relapse, CMV, and EBV-PTLD) that were significant or close to significant ($p<0.1$) in the univariate analysis.

Next we analyzed factors influencing graft quality (those with viability less than 64% in the frozen-thawed vial) using simple regression. We also investigated whether the experimental data from frozen-thawed vials correlated to non-manipulated graft cells just obtained after transport.

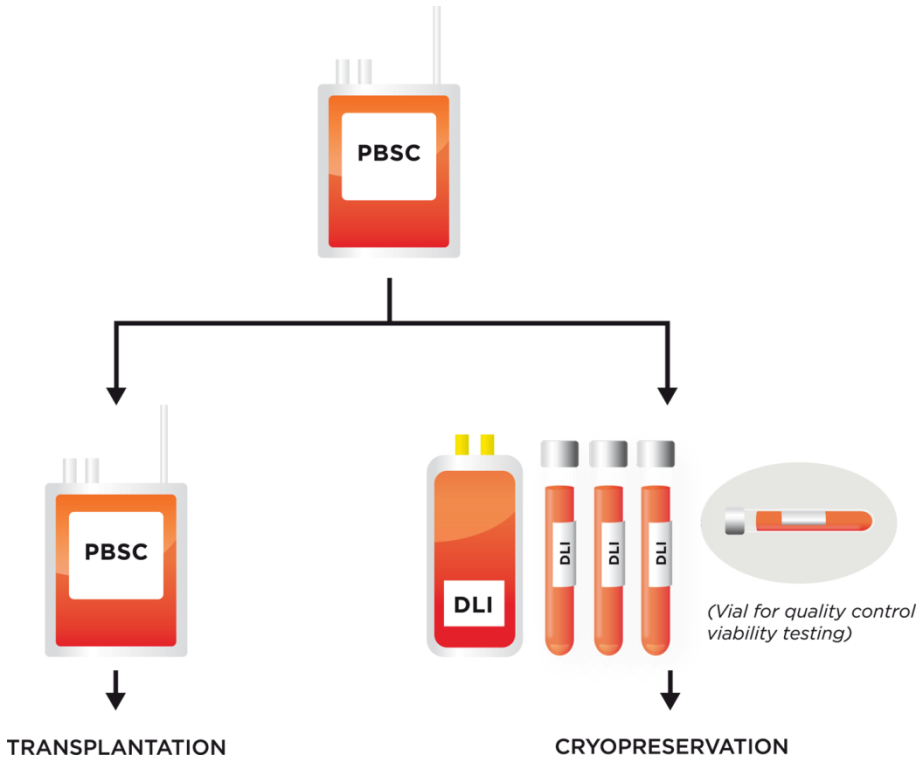

Figure 5: All PBSC grafts were divided into two parts where one was given to the recipient for transplantation. The other part was cryopreserved in escalating CD3+ doses for future donor lymphocyte infusions (DLI). At cryopreservation, a vial for quality control (viability) testing was frozen together with the DLI doses.

3.2.4 Scientific paper IV

In this retrospective, single center study we investigated granulocyte treatments in patients that have undergone allogeneic HSCT. The main objective was to compare effects and adverse events associated with granulocyte transfusions (GCX) between patients who received granulocytes from donors pre-treated with G-CSF and steroids or with steroids only. The indications for GCX treatment were infection with insufficient response to conventional treatment or severe mucositis. One patient received GCX as prophylaxis against infection. To our knowledge, mucositis is an uncommon indication for this treatment and previously not well studied.

Patients who underwent allogeneic HSCT and were treated with one or more GCX at our center between 1998 and 2014 were included in the study. Eighty-seven patients were treated with GCX. Two patients were excluded due to treatment later than a year post-HSCT (5.6 and 3.8 years after transplantation, respectively), resulting in 85 patients eligible for analysis. The median time between HSCT and GCX was 9 days (range 0-240). Malignancies (i.e. hematological neoplasias and solid tumors) were the most common indication for HSCT (76 patients, 89%). The remaining 9 (11%) patients underwent HSCT due to non-malignant

diseases including primary immunodeficiency, inborn metabolic disorders, hemoglobinopathies and bone marrow failure.

GCX treatment indications were severe infection without response to pharmacologic antibiotic treatment (n = 48, 59%), severe mucositis (grade 3-4 according to the WHO classification) (211) (n = 32, 40%) or as prophylaxis in a high-risk patient (n = 1, 1%). In 5 cases the treatment indication was unclear due to missing documentation in the patient chart.

To compare GCX treatment for mucositis to conventional treatment, a group of 32 matched controls with mucositis classified as WHO grades 3-4 who had not received GCX was selected for comparative analysis.

Granulocyte donors were voluntary, eligible, ABO- and RhD compatible blood donors. CMV-negative patients were given granulocytes from a CMV-negative donor. GCSF-GCX donors were pre-treated with 3 mg oral dexamethasone and 0.3 mg G-CSF (filgrastim, Neupogen, Amgen, Munich, Germany) as a single subcutaneous injection 12 hours before collection. S-GCX donors received 100 mg hydrocortisone (Solu-Cortef, Pfizer Inc. New York, USA) as a single intravenous injection 15 min before first collection and then 3 mg oral dexamethasone per 12 hours before subsequent collections.

At our center until 2005 only S-GCX was given. After 2005 G-CSF was added to the mobilization protocol although some GCX after 2005 are S-GCX (steroid pretreatment only). This was either due to time-limitation (collection was performed the same day as requisition) or low recipient body weight.

Leukaphaeresis was performed on CS3000 (Baxter Healthcare, Deerfield, IL, USA) until 2002. From 2003 a Cobe Spectra (Terumo BCT, Lakewood, CO, USA) and the PMN program was used.

Treatment response for infection and mucositis was classified as none, partial or complete, Table 2. Treatment response for the patient receiving prophylactic GCX was not assessed in this study.

Table 2	**Complete Response**	**Partial Response**
Indication:		
Infection	CRP decrease >50% Normalized body temperature Resolution or marked reduction of pulmonary, urinary or local infection symptoms	CRP decrease <50% Improvement but no resolution of symptoms and clinical findings
Mucositis	Resolution of mucositis symptoms or reduction $\geq$ 2 grades on WHO oral mucositis score	Reduction in mucositis related symptoms or reduction 1 grade on WHO oral mucositis score

Table 2: Classification of treatment response after granulocyte transfusions

Mucositis was graded according to the WHO classifications (212).

Adverse events were graded according to a grading system adapted from Sanders et al(213). AEs were classified as febrile, inflammatory, allergic, transfusion-related acute lung injury (TRALI), hypotension, transfusion-related septicemia, hemolysis, circulatory overload or a combination of these. The different AEs were then graded on a scale from 1 to 5, with 1 signifying mild, 2 moderate, 3 severe, 4 life-threatening and 5 fatal.

GCX carries a known risk of serious pulmonary side effects (202, 208). We investigated risk factors for severe pulmonary side effects of granulocyte treatment. To analyze the circumstances of previous pulmonary symptoms and pulmonary AEs, we studied the entire patient cohort for pulmonary radiologic examinations before and after GCX. We divided the patients into three groups before and after GCX depending on pulmonary x-ray findings:

- Patients with pulmonary infiltrates
- Patients with minor pathological findings on pulmonary X-rays (no infiltrates)
- Patients without any pathological findings on pulmonary radiographs

We then studied if patients changed between groups after GCX treatment.

3.3 STATISTICS

Overall survival (OS) was calculated using the Kaplan-Meier method and compared with the log-rank test. Survival time was calculated from the day of transplantation until death or last follow-up. The cumulative incidence of transplant related mortality (TRM), relapse, graft failure (GF) and GVHD were obtained using an estimator of cumulative incidence curves. Patients were censored at the time of death or last follow-up.

Predictive analyses for relapse, TRM, GF and GVHD were performed with the proportional sub-distribution hazard regression model of Fine and Gray.23 Predictive analyses of OS were performed using the Cox proportional hazards model. Logistic regression was used for predictive analysis of CMV infection, PTLD, PRABO, PLS and AIHA. Factors with a p value of equal to or less than 0.10 in univariate analysis were included in the multivariate backwards elimination regression analysis.

Continuous clinical outcome variables were compared with the Mann-Whitney U test or Kruskal-Wallis ANOVA and categorical variables using Fisher's exact test or chi-square test.

Analyses were performed using computer software: cmprsk package, developed by R. Gray, June 2001; S-Plus 6.2 software (Tibco software, US); the Statistica software (Statsoft, US, MA); Microsoft Office Excel 2007 (Microsoft Corp., Redmond, WA); or R Version 3.0.2 [2013-09-25], ("Frisbee Sailing," VC 2013 The R Foundation for Statistical Computing, Platform: i386-w64-mingw32/ i386 [32-bit]).

3.3.1 Scientific paper I

The primary endpoint in scientific paper I was the predictive factors for graft failure (GF).

The predictive factors analyzed for graft failure were ABO-, RhD-, MNSs-, or Kidd mismatch, sex, recipient age, disease stage, TNC dose/kg recipient, CD34+ dose/kg recpient, donor sex, donor age, HLA A, B or DR mismatch, HLA C mismatch, HLA C, DP or DQ mismatch, conditioning regime (busulfan or TBI), ATG type (thymoglobuline or other) and stem cell graft source (bone marrow or PBSC).

3.3.2 Scientific paper II

The clinical outcome and risk factors for patients with PRABO, PLS or AIHA were analyzed in scientific paper II. We also analyzed clinical outcome and transfusion needs dependent on ABO compatibility.

Factors analyzed were ATG, diagnoses, graft type, donor type, disease stage, GVHD prophylaxis, conditioning, GVHD, donor age, granulocyte colony-stimulating factor (G-CSF) after HSCT, herpes virus serology and sex mismatch between donor and recipient.

3.3.3 Scientific paper III

In scientific paper III we analyzed the correlation between the measured graft quality and clinical outcome and infections (CMV, PTLD). Graft quality was assessed by viability testing of control vials from the cryopreserved fraction (the frozen/thawed sample).

Risk factors for clinical outcome analyzed were: recipient age, recipient sex, diagnosis, disease stage, donor type (related or unrelated), HLA mismatch, immune suppressive drugs used, ABO blood group mismatch, CD34 dose/kg recipient, TNC dose /kg recipient, donor age, reduced conditioning regime (RIC), female donor to male recipient, recipient CMV serology positive, acute GVHD I-IV, acute GVHD II-IV, CD3+, CD4, CD16/56+ and CD19+ cell doses/kg recipient.

Correlation between viability measured in the control vials from the cryopreserved fraction and factors influencing graft quality (WBCs, PLTs, CD34, CD3, CD19, CD56/16, time in transit and storage and temperature after transport) were calculated using simple regression or Mann-Whitney U test.

3.3.4 Scientific paper IV

In scientific paper IV we analyzed factors influencing outcome after GCX transfusions in HSCT patients.

Factors analyzed were indication for GCX (infection, mucositis) and donor pre-treatment (steroids only or G-CSF and steroids) and were assessed regarding treatment response, adverse events and clinical outcome.

The effects of GCX on incidence of GVHD, survival, relapse, graft failure were studied.

3.4 AN INTRODUCTION TO THE METHODS USED IN THIS THESIS WORK

3.4.1 Flow cytometry

Flow cytometry is used for analyzing white blood cell markers, stem cells (CD34) and viability (7AAD/CD45) as part of clinical routine. For additional viability assessment of cellular grafts Annexin V has also been implemented at our laboratory.

In flow cytometry analysis particles/cells are labeled using either a dye, such as 7AAD selectively staining DNA in dead or dying cells, or a specific flourochrome-conjugated antibody targeting epitopes on particles/cells. The cells, suspended in a liquid flow, pass a laser and a set of detectors one by one at a rate of up to thousand particles per second. The cells size and granularity are detected as forward and side scatter. If a cell is stained by dye or expresses an antigen targeted by the specific antibody, the flourochrome will emit an energy-pulse when hit by light at a specific wave length from the laser. This energy is detected by the instrument, transformed in to a charge-pulse (mV) which is amplified and displayed in a histogram (with one dimension) or as a plot (showing two dimensions).

The flow cytometer instruments used in this thesis work are FACS Calibur (Becton Dickinson) or FC500 (Beckman Coulter).

3.4.1.1 CD34 analysis

The CD34 analysis at Karolinska is performed using a single platform flow cytometry analysis based on the ISHAGE gating strategy (214). Briefly, about 1×10^6 cells is added to an TruCount™ tube (Becton-Dickinson) and incubated with 7AAD, a CD34-PE conjugated antibody and a CD45-FITC conjugated antibody. The red cells are then lysed before analyzed using a flow cytometer. The ISHAGE (214), serves to reduce variability in the analysis, both within and between laboratories.

3.4.1.2 Viability using 7-Aminoactinomycin (7AAD) and CD45

7-Aminoactinomycin D (7-AAD) is a compound that binds to DNA. It does not readily pass through intact cell membranes hence live cells or cells in early apoptosis will remain unstained. Only dead cells or cells with compromised membranes (permeabilized or disrupted) will be stained. In short, white blood cells are incubated with 7AAD and CD45-FITC conjugated monoclonal antibody. If there are red blood cells present they are lysed using IOTest3 Lysing Solution, prior to analysis on a flow cytometer.

3.4.1.3 Apoptosis and Annexin V

There are a number of different analyses that are used to distinguish cells in apoptosis (215). Annexin V was first described by Koopman *et al* in 1994 (216) as a method for detecting apoptotic cells. In healthy cells the plasma membrane is asymmetrical retaining phosphatidylserine (PS) on the inner leaflet. During the early stages of apoptosis PS is

flipped from the inner leaflet of the plasma membrane to the outer without affecting plasma membrane integrity. Annexin V is an anticoagulatory protein that binds to PS in a Ca^{2+} dependant matter. We use Annexin V conjugated with FITC in an assay combined with 7AAD. In short, graft cells are washed once with a Ca^{2+} containing buffer and centrifuged. The cells are then labeled with Annexin V and 7AAD, incubated, and immediately analyzed by flow cytometry.

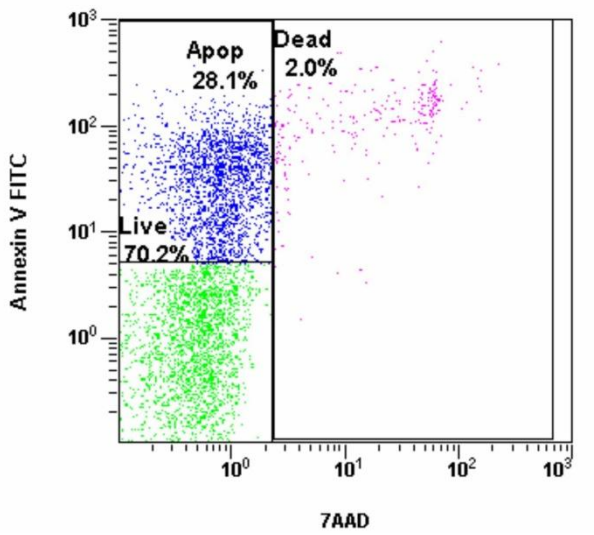

Figure 6: Cells from a PBSC graft are co-stained with Annexin V and 7AAD and analyzed on flow cytometer. First the cells are gated with forward and side scatter. All cells in the white blood cell region are then brought in to the next gate displaying Annexin V and 7AAD shown here. Cells in the lower left quadrant (green dots) are considered to be alive. In the upper left quadrant apoptotic cells binding Annexin V can be seen. Dead cells stain both for Annexin V and 7AAD and are seen in the right (pink dots).

We chose to use Annexin V, out of a number of possible markers of apoptosis, since it is a robust analysis suitable to a clinical routine laboratory. The analysis has one drawback, its calcium dependence (217). All our HSC-products contain citrate as anti-coagulant; the mechanism of citrate is binding calcium. This requires a washing step with calcium buffer to be performed prior to the Annexin V analysis. The validation performed before implementation demonstrated that it did not affect the results (data not shown).

3.4.2 Cryopreservation of HSC and lymphocytes

Several cryopreservation protocols exist primarily differing in cryopreservation solutions and freezing rate (218, 219). Most laboratories use dimethyl sulfoxide (DMSO) as cryoprotectant, suspended in either plasma or human serum albumin (HSA) with heparin. Plasma contains citrate hence additional anticoagulant, such as heparin, is not required. DMSO penetrates the cells preventing intra cellular crystal ice formation protecting the cells from damage when the freezing point is reached. The DMSO concentration is commonly 10 or 5%. The 5%

concentration is shown to be at least as effective (220, 221), if not better improving post-thaw viability (222). DMSO is toxic to non-frozen cells and exposure times should be limited. An additional extra cellular cryoprotectant, i.e. hydroxyl ethyl starch (HES), is added in some protocols (223). HES does not penetrate the cell plasma membrane and is believed to protect the cells by restricting water movement, preventing intra cellular dehydration and protecting the cell against extra cellular ice crystals (218, 223).

When the cells are mixed with cryosolution and put in a cryopreservation bag they should be frozen immediately to avoid DMSO exposure. The freezing rate is important for cell viability (219, 222). Controlled freezing rates using a nitrogen based freezing device can be used, commonly freezing the cells at -1 °C /min the first 40 minutes then -10 °C /min until a final temperature of 80-100 °C is reached. Another option is to use the uncontrolled freezing rate (224-228). In this case the cryobags are put in a -80 °C mechanical freezer for at least two hours and then the cell components are moved to their final storage in liquid-or gas phase nitrogen tanks or low-temperature (<-135 °C) mechanical freezers .

Cryopreserved cells are then stored in tanks containing liquid or gas-phase nitrogen or, in low-temperature mechanical freezers. How long the cryopreserved cells can be stored is not known. However, studies have shown that cryopreserved cells remain viable for at least a decade (229-232). If the cryopreserved grafts are form ineligible donors with positive viral infectious disease markers (such as HBV, HCV or HBV) the grafts cannot be submerged in liquid nitrogen due to risk of transmitting viral disease (233).

At our center we use a plasma and UFR-protocol for cryopreservation of cells. The cells are suspended to equal volumes of a cryopreservation solution (consisting of blood group AB blood donor plasma and 20% DMSO) to a final concentration of 10% DMSO. The cells are then frozen in a -80°C mechanical freezer (UFR) and subsequently transferred for further storage in either liquid- or gas-phase nitrogen tanks or -150 °C mechanical freezers. Cell concentration during cryopreservation is set at a maximum of 200 $x10^9$/L WBC (234, 235).

Cryopreserved cells can be given directly after thawing (no-wash) or thawed and washed. When no wash is performed, the cells are thawed quickly in a 37 °C water bath bed-side and infused immediately to the patient. If the cells are washed prior to infusion, the cryopreserved cells are thawed quickly in a 37 °C water bath at the cell processing laboratory and washed, by a dilution/centrifugation step, before distributed to the ward and given to the patient. The washing procedure removes most of the DMSO from the cell component reducing DMSO toxicity in the patient (236, 237). At our center all autologous HSC to adult patients are washed prior to infusion (238). Other cells are thawed bed-side and directly infused.

3.4.3 Aphaeresis

The word aphaeresis means taking away and in this context aphaeresis is used to separate blood into components. This is done by centrifugation or filter/adsorption columns. Aphaeresis can be used to collect cells or for therapeutic reasons.

Filter columns are, for example, LDL-filters used to remove low-density lipoprotein (LDL) from plasma in patients with severe inherited hypercholesterolemia, Adacolumn filters that remove nucleated cells in patients with inflammatory bowel disease, or columns removing antibodies, by targeting either the Fc-part of the antibody and thus removing immunoglobulin's in general or by targeting the variable part of the antibody removing antibodies of a certain specificity such as ABO antibodies in major ABO incompatible organ transplantation.

In leukaphaeresis a centrifuge technique is used to separate blood based on the density of each cell. The densities of different cell types are depicted in figure 7.

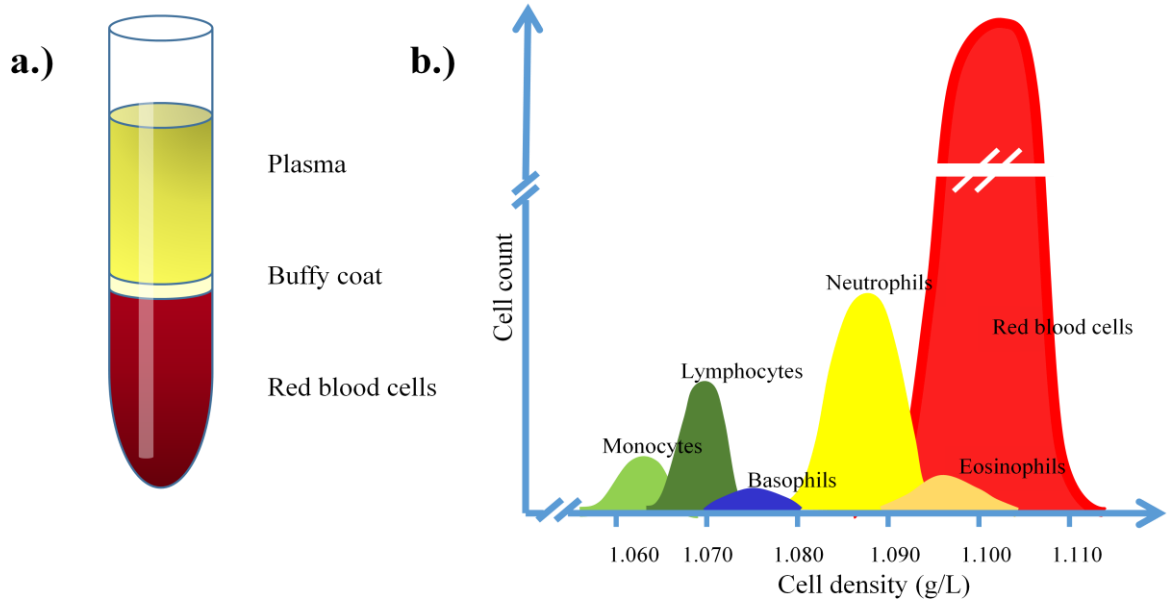

Figure 7: a.) The elements of whole blood separate according to density when exposed to centrifugation. b.) The cell count in whole blood and the density of the different blood cells and are visualized.

Briefly, blood is drawn at an access point from a peripheral or central vein and led into the centrifuge in the aphaeresis device. The blood is separated and the desired blood fraction (in leukaphaeresis it is a fraction of the white blood cells) is collected and led in tubings into the collection bag. The rest of the blood is returned to the donor/patient through a return point in a peripheral vein (usually the other arm) or to the central vein. Any fraction of the blood can be collected or removed using a centrifuge aphaeresis device. Different anticoagulants can be

used, ACD-A, sodium citrate, CPD or heparin. The amount of blood processed through the device depends on how much cells or plasma needs to be collected or removed.

Figure 8: Apheresis devises; Spectra Optia and Cobe Spectra (Pictures of aphaeresis devises: Copyright, Terumo BCT, Inc. Used with Permission)

3.4.4 Analysis of Blood group and antibodies against blood group antigens

A persons ABO blood group is determined by analysis of ABO antigens on red blood cells (direct typing) and the presence of anti-A or anti-B in plasma (reverse typing) according to routine methods using an automated system (AutoVue; Ortho Clinical Diagnostics, Raritan, NJ) or manually using tube or gel techniques (Bio-Rad Laboratories, Herts, UK) (239).

Screening for irregular antibodies or auto-antibodies is performed using the indirect anti-globuline test (IAT) with gel technique (ID-Coombs Anti-IgG; Bio-Rad Laboratories) or with the AutoVue system utilizing antihuman globulin, (AHG) anti-IgG.

Direct anti-globulin test (DAT) was analyzed using a manual tube method (239) or a gel technique (ID-Liss Coombs, DC-Screening I; Bio-Rad Laboratories). After 2005 the automated AutoVue system (Ortho Clinical Diagnostics) was used.

In patients with a positive DAT the antibodies can be eluted in order to determine their specificity by IAT in gel technique. The elution method used is cold acid elution with ELU-KIT II (Gamma Biologicals, Houston, TX).

In major ABO mismatched HSCT the anti-A or –B titer of the recipient is determined using tube or gel techniques (239). If donor RBCs are available these are used for titer determination, otherwise a test cell RBC of the same ABO blood group is used.

Chimerism of RBCs is assessed by RBC typing of the donor and the recipient prior to HSCT defining a marker, a difference in blood group between donor and recipient. After HSCT this difference in blood group can be used to estimate the proportion of donor- or recipient type red blood cells in the recipients' blood. This is performed by blood typing using tube technique and monoclonal antibodies. After incubation and centrifugation the reaction is read and the percentage free RBCs are assessed using a microscope.

4 RESULTS AND DISCUSSION

4.1 BLOOD GROUPS IN HSCT (SCIENTIFIC PAPERS I AND II):

Scientific paper I and II were performed as single center retrospective studies studying the impact of blood groups in allogeneic HSCT. Scientific paper II has two perspectives; one is the impact of ABO differences in donor and recipient and their implication on clinical outcome. The other perspective is the impact of detected ABO antibodies. The results from scientific paper I and II with regards to the impact of blood groups on clinical outcome will be presented and discussed together (Part 1). The impact of detected ABO antibodies will be discussed separately (Part 2).

4.1.1 Part 1: The impact of blood groups on clinical outcome after allogeneic HSCT

4.1.1.1 Results of scientific paper I: Major ABO Blood Group Mismatch Increases the Risk for Graft Failure after Unrelated Donor Hematopoietic Stem Cell Transplantation

Among the 224 patients included in this study 89 (40%) patients received an ABO identical graft, 67 (30%) received a major ABO mismatched graft and 68 (30%) were minor mismatched. A bidirectional mismatch was found in 16 (7%) cases.

Graft failure (GF) occurred in 6 (2.7%) patients with a median of 64 days (range 23-132) after HSC; 3 had primary GF and 3 secondary GF. The only factors significantly associated with GF were ABO mismatch (odds ratio [OR] 14.9, 95% confidence interval [CI] 2.01-110, p =0.008) and HLA-A, -B, or -DR allele level mismatch (6.42, 1.19- 34.8, p=0.03). In patients with and without major ABO mismatch, the incidence of GF was 7.5% (5 of 67) and 0.6% (1 of 157), (p=0.02) respectively. In patients with and without HLA allele level mismatch, the incidence of GF was 8.3% (2 of 24) and 2.0% (4 of 200) (p=0 .09), respectively. In patients with both a major ABO mismatched and an HLA-allele mismatched donor (n = 5), the incidence of GF was 20%, figure 9. None of the patients without risk factors developed GF.

In this study the main graft source was bone marrow. Removal of RBCs from ABO major mismatched bone marrow (BM) grafts led to a decrease in total nucleated cell (TNC) count by 20%. However, there was no statistical differences between the TNC counts in ABO major mismatched BM grafts (after RBC removal) with all ABO non-major mismatched BM grafts (no RBC removal), (2.5 [range 0.2-17.0] versus 2.6 [range 0.7-13.1], p=0.5). Furthermore, graft failure was not affected by cell counts in BM grafts ABO major mismatch (with or without GF, 2.2 [range 0.7-14.0] versus 2.5 [range 0.2-7.0], p=0. 8).

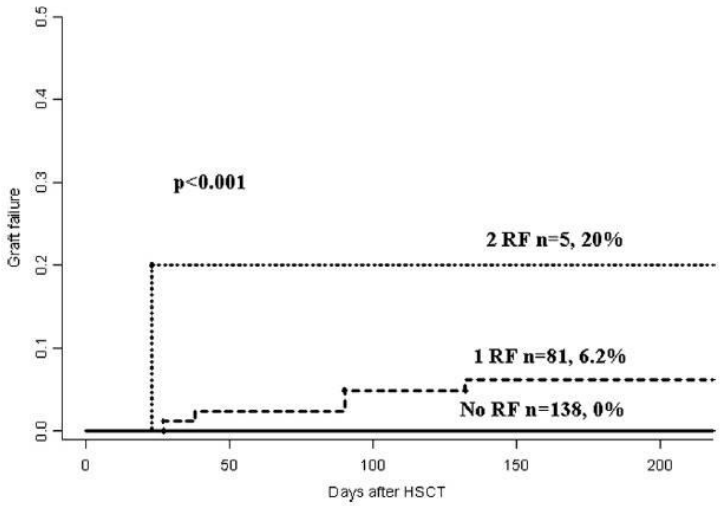

Figure 9: Cumulative incidence of graft failure (GF) depending on number of risk factors (RF) found in the multivariate analysis. Risk factors were ABO major mismatch and HLA allele mismatch).

There was no difference in pre-HSCT ABO antibody titers between patients with and without GF in major ABO mismatched transplants. The IgG antibody titers were 16 (8-128) and 32 (1-1000), the IgM titers 8 (range 1-32) and 32 (range 1-1000) in patients with and without GF, respectively (Ns).

A blood group antigen RhD mismatch was found in 53 (24%) cases, a MNSs mismatch in 144 (64%), and a Kidd (Jka/b) mismatch in 99 (44%) cases. No correlation between other blood group antigens (Rh, MNSs, and Kidd) and graft failure was found.

4.1.1.2 Results of scientific paper II: Analysis of Donor and Recipient ABO Incompatibility and Antibody-Associated Complications after Allogeneic Stem Cell Transplantation with Reduced-Intensity Conditioning

Among the 310 patients included in this study 145 had an ABO identical donor, 66 received grafts from a minor ABO mismatched donor and 95 had a major or bidirectional ABO mismatched donor. No significant differences were observed between the ABO-matched and –mismatched groups in overall survival (OS), transplant related mortality (TRM), acute GVHD grades II to IV, chronic GHVD, or graft failure by multivariate analysis, Table 3.

Outcome	AB0 identical (n=145)	Minor AB0 MM (n=66)	Major AB0 MM (n=95)	
OS	63%	53%	66%	Ns
RFS	68%	67%	76%	Ns
TRM	23%	26%	24%	Ns
aGVHD II-IV	27%	30%	38%	Ns
cGVHD	37%	29%	29%	Ns
GF	18/145, 12%	7/66, 11%	10/95, 11%	Ns

Table 3: The impact of ABO match on clinical outcome (scientific paper II suppl.table).

4.1.1.3 Discussion: Impact of blood group antigens on clinical outcome

In scientific paper I we found a correlation between major ABO mismatch, especially in combination with HLA-mismatch, and graft failure. In contrast to the results in scientific paper I we found no correlation between ABO mismatch and graft failure in scientific paper II. The two studies differ in study population and in date of transplant. In scientific paper I we studied 224 patients with leukemia receiving full myeloablative conditioning between 1991-2003, whereas in paper II patients receiving RIC treatment in 1998-2011 where studied. At our center graft failure has remained at the same level over this whole period (39), thus excluding different time periods as explanation for the difference between these two studies. ABO major mismatch was also identified as a risk factor for GF in o another study (Remberger *et al*) at our center (240) but in the study by Olsson *et al* (241) ABO mismatch was significant in the univariate analysis but not in the multivariate analysis.

A large observational study by Seebach *et al* (CIBMTR database) including over 3000 patients with leukemia showed no impact of ABO differences between donor and recipient on survival, TRM, relapse or chronic GVHD (180). In this study they did find a prolonged engraftment of, both neutrophils- and red blood cell engraftment. The latter was measured as prolonged transfusion dependence. Additionally, the patients receiving bidirectional ABO mismatch had more severe acute GVHD (grade III-IV), but given there was no influence on TRM or OS, these results should be interpreted with caution. The graft sources were bone marrow. Although the transplanted cell dose did not differ between the groups in scientific paper I or in the study by Seebach *et al* (180), the grafts given in major and bidirectional ABO mismatched patients were most probably red blood cell reduced. All interventions with bone marrow grafts affect cell dose (121) or compromise cell composition which may influence engraftment and risk of graft failure.

In a recent study by Olsson *et al* 2015 risk factors for GF were analyzed in 1278 patients with reported GF out of 23 000 patients in the CIBMTR database (242). Here, major ABO mismatch was identified as a risk factor for GF (both in HSCT with related or unrelated donor and independent of BM or PBSC graft source). Major ABO mismatch remained a risk factor when adjusting for cell dose.

The association between GVHD and minor ABO has been described by others as reviewed by Stussi *et al* (243). It is speculated that donor type ABO antibodies bind recipient endothelial ABO antigen, causing tissue damage and evoking an acute GVHD reaction. This could not be confirmed in our study, scientific paper II, nor in the Seebach study (180).

The impact of ABO on clinical outcome after allogeneic stem cell transplantation is still debated (175, 180, 243-245). Reported results are conflicting but these studies do differ in patient cohorts and in treatment- and transfusion regimes. The effect of ABO may become more apparent as transplant techniques advances and transplant indications change. Other factors of greater impact may override the effect of ABO donor-recipient differences thus obfuscating its influence and complicating analysis and the interpretation of results.

4.1.2 Part 2: ABO antibodies in allogeneic HSCT, complications and effect on clinical outcome

4.1.2.1 Results of scientific paper II: Analysis of Donor and Recipient ABO Incompatibility and Antibody-Associated Complications after Allogeneic Stem Cell Transplantation with Reduced-Intensity Conditioning

In scientific paper II we investigated hemolytic complications that could arise from ABO-incompatible transplants; passenger lymphocyte syndrome (PLS), persisting or recurring recipient ABO type antibodies (PRABO) and also autoimmune hemolytic anemia (AIHA) and their impact on clinical outcome. The results are summarized in Table 4. We also analyzed transfusion requirements with regards to ABO differences between donor and recipient.

Outcome	**PLS**		**AIHA**		**PRABO**	
	Yes	**No**	**Yes**	**No**	**Yes**	**No**
OS	0%	61% ***	72%	62%	17%	73% **
RFS	0%	75% **	75%	70%	33%	78% *
TRM	33%	22%	19%	24%	50%	21% *
aGVHD II-IV	50%	32%	0%	33% *	51%	30%
cGVHD	0%	34%	13%	34%	25%	34%

Table 4: Outcomes in PLS, PRABO and AIHA patients. * p<0.05, ** p<0.01, *** p<0.001

Passeger lymphocyte syndrome (PLS)

In this study we defined PLS as the presence of donor type anti-A and/or anti-B antibodies either on the recipient's RBCs or in recipient plasma during the first month after minor ABO mismatch HSCT. Based on these criteria, we found 6 patients that had PLS. Survival in PLS patients was 0% compared to 61% of patients without PLS (P <0 .001) (Figure 10 A). There was no difference in TRM in PLS patients compared to patients without PLS (33% versus 22%, ns). Risk factors for PLS in univariate analyses were transplants with unrelated donors (p=0 .02), the absence of methotrexate (p=0.03), and nonmyeloablative conditioning for solid tumor (p=0.016). A multivariate analysis was not possible since all PLS patients had an unrelated donor and had received ATG. The causes of death in PLS patients were progressive disease of solid tumor (n = 4) and pneumonia (n = 2). The graft source did not affect PLS incidence.

Recurring recipient type ABO antibodies (PRABO)

Of the 95 patients receiving a major ABO mismatched HSCT, 12 patients had anti-A/B antibodies detectable >3 months post-transplant and hence were classified as individuals with persistent or recurring recipient type ABO antibodies (PRABO). Patients with PRABO had more complications post-HSCT. They had a greatly decreased 3-year survival (17% versus 73%, p=0 .002) (Figure 10 B), a significantly higher TRM (50% versus 21%, p=0.03), and more RBC and platelet transfusion needs compared to patients without PRABO receiving a major ABO mismatched HSCT.

The clinical picture of these 12 patients is as follows. Three had a picture consistent with pure red cell aplasia (PRCA), that is, low reticulocyte count in peripheral blood, normal lactate dehydrogenase, and normal or subnormal levels of platelet. Two patients had normal or high cell content in the bone marrow, a normal erythropoiesis, normal or high levels of reticulocytes in peripheral blood, low number of platelets and high levels of lactate dehydrogenase. Hemolysis resolved in these 2 patients without other treatment than ABO-compatible RBC and platelet transfusions. The remaining 7 patients presented with varying degrees of hemolysis, poor marrow function with mixed chimerism, and/or cytomegalovirus infections. In four of these patients, transplant indication was solid tumors. Two also had unspecific anti-RBC auto-antibodies and HLA antibodies.

The use of G-CSF treatment of the patients post-HSCT was the only significant risk factor identified by univariate analysis for PRABO after major ABO mismatch HSCT (p=0.04). There was also a trend for patients treated for solid tumors (p=0.06). PRABO incidence was independent of the graft source used.

We found no correlation between PRABO and anti-A/anti-B antibody titers in recipient plasma prior to HSCT.

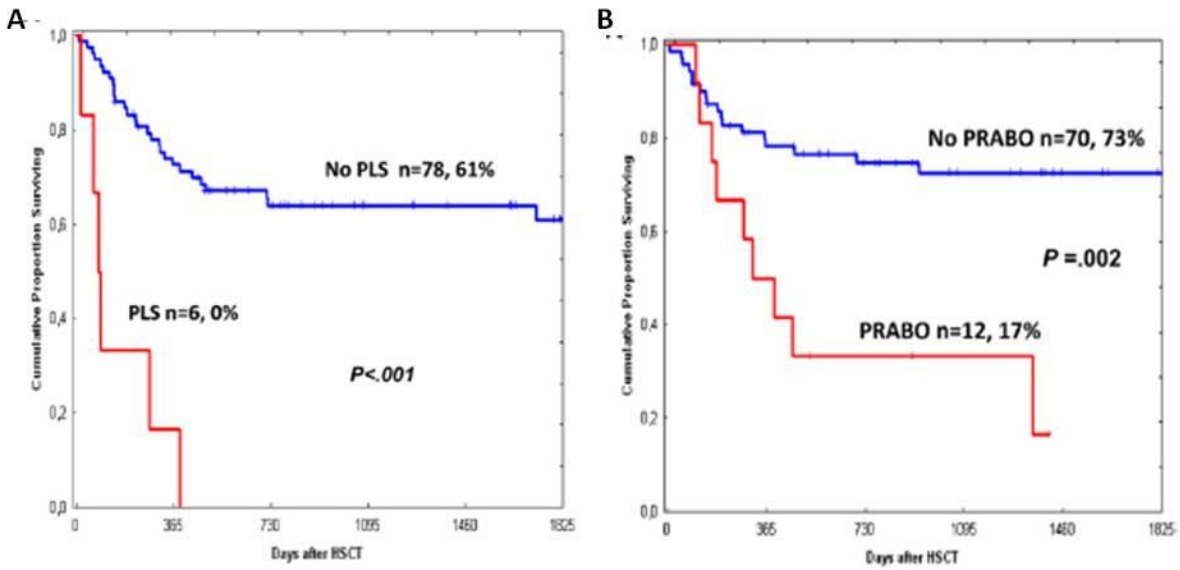

Figure 10: Cumulative survival in A) patients with or without PLS in minor ABO transplants and, B) patients with and without PRABO in major ABO transplants. Patients with PLS or PRABO have significantly lower survival.

Autoimmune hemolytic anemia (AIHA)

In scientific paper II we defined AIHA as patients with positive direct anti-globulin test (DAT) and positive indirect anti-globulin test (IAT) after transplantation. The antibodies should be non-ABO specific antibodies. We identified 12 (4%) patients with AHIA after HSCT. There was no association between AIHA and a decrease in OS or increase in TRM. However, patients with AIHA did not experience any acute GVHD grades II to IV, which was significantly lower than patients without AIHA (p=0 .04).

Risk factors for AIHA identified by univariate analysis were; use of ATG ($p < 0.01$), stem cells from unrelated donors (p=0 .047) and younger donor age (<38 years) (p=0.03). We also found that patients seropositive for 0 to 2 herpes viruses before HSCT were more likely to develop AIHA than those positive for 3 to 4 herpes viruses (p=0.02). The incidence of acute GVHD was lower in AIHA patients than patients without AIHA (p=0 .006). In the multivariate analysis, only patient virus serology before HSCT was significantly associated with AIHA (odds ratio, .22; 95% confidence interval, 0.06 to 0.8; p=0.02). The use of an unrelated donor had a strong trend as risk factor for AIHA (odds ratio, 7.38; 95% confidence interval, 0.92 to 59.5; p=0.06). It was not possible to analyze the effect of ATG and acute GVHD since all AIHA cases received ATG and had no acute GVHD. The graft source used did not affect the incidence of AIHA.

There was a higher prevalence of positive DAT after HSCT in patients receiving ABO-non identical, rather than ABO –identical, graft HSCT (p=0.001). A positive DAT in itself did not significantly affect outcome of OS, TRM, and graft failure.

Transfusion requirements after allogeneic HSCT

Patients with minor and major ABO incompatibility required significantly more RBC transfusion at both 2 and 12 months post-HSCT compared with the ABO-matched group (Table 5 A). Patients with major ABO mismatch also required more platelet transfusions. In addition, patients with PRABO needed more transfusions than patients where no persistent recipient type ABO antibodies had been detected (Table 5 B).

Table 5 A	**RBC+2 months**	**PLT+2 months**	**RBC+12 months**	**PLT +12 months**
ABO Identical	2 (0-34)	1 (0-48)	4 (0-98)	4 (0-77)
ABO Minor mismatch	4 (0-50) **	1 (0-31)	9 (0-88) **	3 (0-74)
ABO Major mismatch	5 (0-45) ***	2 (0-41) *	8 (0-282) **	4 (0-115)
Kruskal-Wallis	p<0.001	p=0.08	p=0.002	p=0.19

Table 5 B	**RBC+2 months**	**PLT+2 months**	**RBC+12 months**	**PLT +12 months**
No recipient type ABO ab (n=70)	4 (0-45)	2 (0-27)	6 (0-282)	2 (0-115)
PRABO (n=12)	11 (0-42)	6 (0-41)	44 (6-92)	14 (0-70)
Kruskal-Wallis	p= 0.013	p=0.06	p<0.001	p<0.001

Table 5: ABO incompatibility and transfusion requirement. A.) Transfusion requirements in ABO minor or major mismatch versus ABO identical. B.) Transfusion requirements in major ABO mismatch in patients with or without PRABO.
Numbers indicate the median number of units transfused per patient during the time period stated. PLT indicates platelet transfusions and RBC red blood cell transfusions.
*** p<0.05, **p<0.01, ***p <0 .001 versus ABO match.**

4.1.2.2 Discussion: Clinical effect of PLS and PRABO

In scientific paper II we focused on patients receiving reduced intensity conditioning since this conditioning regime does not totally eradicate recipient immune cells, enhancing the risk of antibody mediated complications (179, 246).

The occurrence of PLS after HSCT is described by several centers. However, some centers report frequent PLS after HSCT (178, 179, 247), while others, like us, and rarely see this phenomenon (scientific paper II). At our center we screened for PLS using DAT and reversed typing at every blood requisition from day 0 to one month post-HSCT. This minimizes missing patients in the study strengthening the incidence analysis. We identified 6 patients with donor ABO antibodies after HSCT out of 66 minor ABO mismatch patients, thus meeting our criteria for PLS in this study. None of the patients displayed clinical hemolysis.

PLS has long been reported in organ transplantation and the risk is related to the amount of lymphoid tissue the organ contains and the use of cyclosporine alone as immune suppressant (without metothrexate) (176) PLS with high frequency after HSCT have been reported (178, 247-249). Worel *et al* reported an incidence of 4 out of 11 patients receiving minor/bidirectional HSCT. The patients in this study had hemolysis that was treated by red blood cell exchange. After 2001, this center performed prophylactic red blood cell exchange for all patients undergoing minor ABO mismatched transplants with a donor-against-recipient type ABO titer over 32. Bolan *et al* reported of massive hemolysis treated with compatible transfusions. Both of these studies reported death related to hemolysis.

Previously reported risk factors for PLS in HSCT are immunosuppression using cyclosporine without methotrexate, the use of PBSC and reduced intensity conditioning (181, 248, 250). The absence of methorexate was confirmed as a risk factor for PLS in our study (scientific paper II) as was non-myeloablative conditioning and use of unrelated donors. Methotrexate was not used in the Bolan study and in the Worel study, MFF was given but the authors speculate in a later paper that the MFF dosage was probably insufficient (178). Increased vigilance by the transplant physician for signs of hemolysis is warranted if an immune suppression regime is used that does not use methotrexate or an equivalent B-cell suppressing drug.

Our study showed that few patients had donor ABO antibodies with little to no clinical hemolysis at our center. As a direct result of this study, standard operating procedures at the blood bank, in consensus with the HSC transplant physicians, were changed removing the requirement for DAT and reversed typing in all blood requisitions in minor ABO-mismatched HSCT during the first month post-HSCT.

In scientific paper II, PLS was associated with poor survival. This was not due to hemolysis, which was minor or non-detectable. However, four out of six patients died due to relaps/progression in solid organ tumors where overall results are generally poor.

Zaimoku *et al* reported that regular ABO antibodies and PLS preceded the onset of acute GVHD (247). In their study 6/18 patients developed PLS and moderate hemolysis. All PLS patients developed acute GVHD, compared to 3/13 in the non-PLS patient group. Also survival was poor and TRM high (4/5 PLS patients died). This was also described in a case report by Salmon *et al* (251). This suggests that antibodies may play a role in the development of acute GVHD. Of note though, the donor cells in the Zaimoku study were cryopreserved before HSCT. Cryopreservation may alter cell composition. A relation between PLS or minor ABO mismatched HSCT and acute GVHD could not be confirmed by our study (scientific paper II).

In major ABO mismatched transplants delayed hemolysis or pure red cell aplacia (PRCA) can occur, usually months after the transplantation. These conditions are attributed to recipient type ABO antibodies targeting antigens on donor red blood cells (181). In delayed hemolysis the patient presents with high or normal reticulocytes, high lactate dehydrogenase (LD), normal cell count in bone marrow samples and normal or vivid erythropoiesis. In our study we found two PRABO patients with delayed hemolysis. Both patients received RBC and platelet transfusions and eventually the condition resolved. Patients can also develop PRCA, a condition with low reticulocytes, low erythroid precursors but adequate myeloid, lymphoid and megakaryocyte cell lines in bone marrow, donor cell types in white blood cell chimerism and transfusion dependence. It is speculated that the recipient anti-donor-ABO antibodies enter the bone marrow and destroy erytroid precursors (181) but the mechanism behind PRCA is not fully known.

It has been proposed that reduction of pre-transplant ABO antibody titers could reduce incidence of PRCA leading to faster red cell engraftment (252-254). PRCA develops later in the post-HSCT process and considering that there is no correlation between pre-HSCT anti-A and/or anti-B titer and PRABO (scientific paper II) or PRCA (255) there is not an obvious pathophysiological explanation for an intervention that reduces pre-transplant antibodies. Additionally, it is reported that there is no correlation between ABO-titers and level of plasma cell chimerism in peripheral blood (54).One can speculate that recipient ABO antibodies have an adverse effect on donor erythroid progenitor cells at the time of transplant inflicting harm that lingers. However, PRCA subsides when persisting recipient ABO antibodies diminish months after HSCT, speaking against such a hypothesis (255). Risk factors for PRCA are not fully known but the use of non myeloablative conditionings may enhance the risk for PRCA (246, 256). The only risk factor for PRABO in scientific paper II was the use of G-CSF.

It has been shown that engraftment of erythrocytes measured as increased transfusion dependence, is prolonged after major ABO mismatched transplantation (175, 180) and that this seems to be independent of transplantation cell dose. This was confirmed in our study (scientific paper II). PRABO patients also needed more transfusions than patients without PRABO. This further implicates involvement of ABO immunoglobulins causing increased transfusion requirements. Blin *et al* (175) showed that ABO regular antibodies disappeared

faster after HSCT from unrelated donors related donors. They also showed that patients with grafts from related donors experiencing acute GVHD grade III-IV cleared the recipient type ABO antibodies faster than the patients without acute GVHD. This indicates that donor – recipient disparity influences persistence of recipient ABO antibodies. The use of non-myeloablative conditioning has also been shown to give longer erythroid engraftment and prolonged persistence of ABO antibodies in major ABO mismatched transplants (246).

In scientific paper II we found that patients with minor or major ABO mismatches also required more platelet transfusions, as did the two patients with PRABO that presented with hemolysis but normal erythropoiesis. We speculate this due to platelets also expressing ABO-antigens.

In conclusion, differences in recipient-donor ABO blood groups can give rise to different complications presumably due to antibodies. The cause, mechanism and effect of the complication depend on when the complications occur in relation to the time of transplant. These complications can be divided into three groups; immediate, early and later. The presences of ABO antibodies can also be associated with clinical outcome factors such as OS, TRM and GVHD. Several interventions and treatment options have been proposed and reported. The impacts of these are difficult to assess due to the differences in complication frequencies between centers and small study population cohorts. The complications discussed and the proposed interventions are summarized in Table 6.

Table 6	**Immediate** Day 0: during or after HSCT	**Early** Day +7-14 (-30)	**Later** Day +90	**Outcome effects** Any time point post HSCT
Type of complication	Adverse events (AE) during HSC infusion	Hemolysis of recipient type RBC	Hemolysis of donor type RBC	Reported as risk factor for:
Mechanism in:				
Minor ABO	ABO incompatible red blood cells in BM	PLS (Donor derived antibodies against recipient antigen)		Acute GVHD (243)
Major ABO	ABO incompatible plasma in BM or PBSC		PRABO (Recipient antibodies against donor antigen) causing PRCA or delayed hemolysis	Prolonged red cell engraftment (180, 248) Prolonged neutrophil engraftment (180) GF (242, 257)
Bidirectional	ABO incompatible plasma and RBC in BM or PBSC	PLS (Donor derived antibodies against recipient antigen)	PRABO (Recipient antibodies against donor antigen) causing PRCA or delayed hemolysis	Acute GVHD (180)
Interventions:				
Conventional Interventions	Depletion of incompatible RBC and/or plasma in HSC graft	Transfusion with compatible blood	Transfusion with compatible blood	
Proposed alternative or additional interventions:	Reduction of ABO antibodies in recipient plasma prior to HSCT by: - TPE or antigen/Ig specific columns) (253) - Transfusion of donor type plasma or RBC (252, 254)	Pre-emptive RBC exchange (178)	TPE or Ig immune adsorption (258), anti-thymocyte globulin, erythropoietin, corticosteroids, rituximab, DLI Tapering of Immune suppressive drugs (255)	Avoiding ABO incompatibility between donor and recipient if possible. Plasma depletion of graft and avoidance of donor type plasma products (247)

Table 6: Summary of ABO-antibody related complications after allogeneic HSCT. The complications are shown in separate columns as immediate, early, and late complications, and transplant outcome. For each complication the mechanisms behind and conventional as well as proposed interventions are summarized.

4.1.2.3 Discussion: AIHA after allogeneic HSCT

AIHA post-allo HSCT is a condition independent of ABO match between donor and recipient, where donor derived antibodies reacts against donor antigens. The incidence of AIHA was 4% in our study, which is in agreement with other reports (186, 187, 259). AIHA did not provide an increased risk for TRM or inferior OS which have been reported by others (186, 187, 259). This may be due to differences in AHIA definition in the different studies but could also potentially be due to differences in conditioning regimes and/or immunosuppressant.

In our study, identified risk factors for development of AIHA were unrelated donors and patient sero-positivity for less than 3 herpes viruses. Patients with sibling donors, absence of both ATG and acute GVHD, had a decreased risk of AIHA development. Previous studies reported that unrelated donors confer an increased risk of AIHA (187, 259). The use of unrelated donors and virus serology was also significant in multivariate analysis in our study. Because all unrelated donors were treated with ATG, it was not possible to analyze this factor in multivariate analysis.

Viral reactivation after HSCT is primarily controlled by natural killer cells and effector T cells. In our study, patients with positive serology before HSCT for 0 to 2 herpes viruses compared with 3 to 4 had a higher risk of AIHA. As a consequence, individuals with less prior virus exposure will have a higher risk of a primary infection after HSCT. It has been shown that a strong T cell-mediated immune activation can lead to autoimmune diseases due to both molecular mimicry and bystander activation (reviewed in (260)).

Interestingly, the 12 AIHA patients had a significantly lower risk of developing moderate-to-severe acute GVHD. That all patients with AIHA lack the occurrence of acute GVHD is difficult to explain. It can be speculated that either the acute GVHD per se or the following corticosteroid treatment and prolonged immune suppression inhibited B-cells responsible for AIHA, but no literature on this topic can be found. Although ATG and acute GVHD could not be analyzed by multivariate analysis, it might also be possible that acute GVHD is merely dependent on the presence of ATG.

For the association between GVHD, herpes virus serology and AIHA, we can speculate that among herpes viruses, EBV is harbored in B cells. Herpes virus antigens may also be a stimulus for alloreactivity with induction of acute GVHD and the corresponding graft-versus-autoimmune effect with elimination of antibody-producing cells and hence decreasing the risk

4.2 VIABILITY AND GRAFT QUALITY

4.2.1 Scientific paper III Quality of the hematopoietic stem cell graft affects the clinical outcome of allogeneic stem cell transplantation

We have observed that the viability of WBCs in frozen-thawed samples, measured by 7AAD/CD45, has a much wider spread than the viability of the non-cryopreserved graft observed at the time of arrival. We speculated that the thawed vial could be used as a "stress test" for the graft and give a better indication of its quality than viability measured on fresh cells after transport.

Graft quality and effect on clinical outcome

In this study the median viability of the frozen-thawed sample was 64%. We set this median as a cutoff and divided the patients into two groups, those with inferior graft quality (viability <64%) and those with better quality (viability>64%), enabling us to analyze the impact of graft quality on clinical outcome.

We found that acute GVHD of any grade was more prevalent in patients receiving PBSC grafts of low quality than those of better quality (72% vs. 57%, p=0.02; aGVHD Grade 0 [n=52], Grade I [n=31], Grade II [n=49], Grades III-IV [n=12]). Transplant related mortality (TRM) was also significantly higher in patients receiving grafts of inferior quality (22% vs. 8%, p=0.03). The graft quality did not significantly affect the incidence of OS or relapse. Figure 11.

There was no difference in engraftment measured as numbers of platelets (PLT)>50 × 10^9/L and neutrophil concentration (ANC) >0.5 × 10^9/L in peripheral blood. Similarly, there was no difference in the number of graft rejections between patients receiving grafts of inferior or better graft quality, n=4 (5.3%) and n=2 (2.9%) respectively (p=0.68).

In our study, both acute GVHD and TRM were correlated with inferior graft quality, and no difference was observed in engraftment time or OS (Figure 11). Our data suggest that engraftment and OS, clinical variables that are often used to assess graft quality, may not be of adequate sensitivity to be the sole factors used for quality assessment of grafts.

We found a correlation between acute GVHD and graft quality measured as viability <64% on frozen-thawed sample. Lazarus *et al* (261) did not find any association between the time in transit and acute or chronic GVHD. These conflicting results might be due to that assessment of graft quality in our study was more a test of general quality of the cells rather than the impact on GVHD by a single variable (transport time).

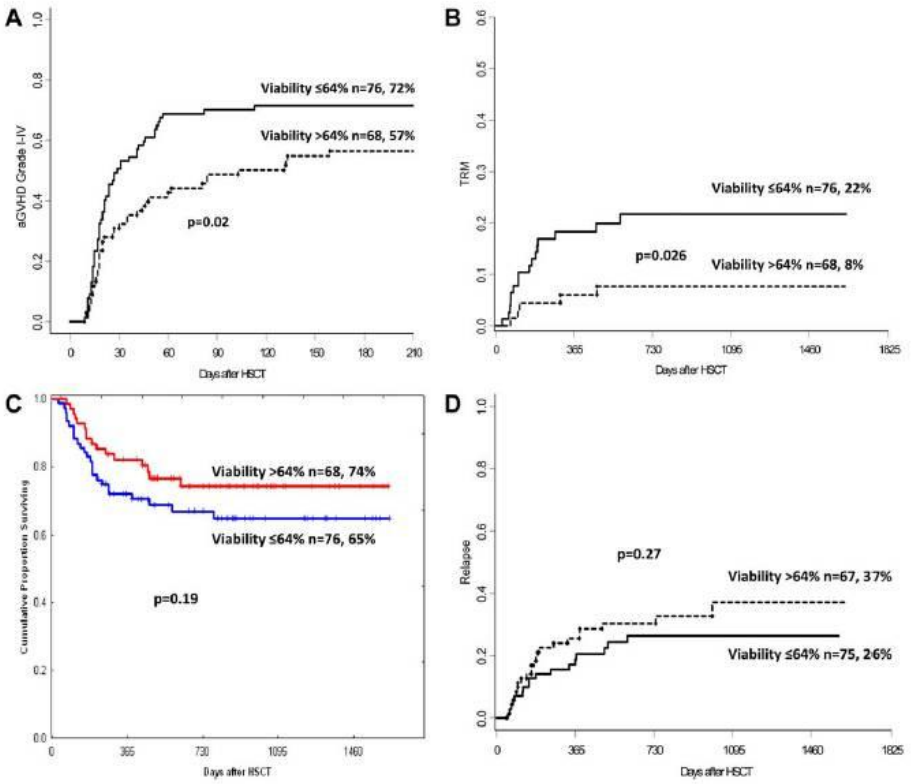

Figure 11: The effect of graft quality on clinical outcome after HSCT. The cumulative incidence for a specific outcome variable is plotted as a function of patients receiving PBSCs with inferior (viability <64% in the frozen-thawed sample) or better (viability >64% in the frozen-thawed sample) graft quality. Outcome variables shown are the cumulative incidence of (A) acute graft versus host disease (acute GVHD); (B) transplant related mortality (TRM); (C) overall survival (OS) and (D) relapse.

The dose of CD34+ cells and total nucleated cells did not affect clinical outcome in this study, nor did the CD3+, CD4+, or CD19+ cell dose. However, a higher natural killer (NK) cell dose was associated with less CMV infection (p=0.03 in the multivariate analysis).

There is a known association between NK-cell biology and CMV infection (reviewed in Della Chiesa *et al* (9)). Studies have shown a protective role of certain NK-cell subpopulations against CMV reactivation and infection (10, 69). Others have shown a clear effect in the other direction, of CMV reactivation modulating the NK-cell repertoire in healthy individuals and after HSCT (10, 262). Studies have shown an impact of the graft composition on different clinical outcomes (263-265) but to our knowledge no studies have shown a correlation between a protective effect against CMV and the NK-cell dose in the graft.

Factors affecting graft quality

In our study the PBSC grafts with inferior quality (<64% viability of the frozen-thawed sample) had significantly higher WBC and PLT concentrations compared to grafts with better quality (viability >64%),(Figure 12). The CD34+ concentration was higher in the inferior quality grafts (median 2055 $\times 10^6$/L vs. 1590 $\times 10^6$/L, respectively; p<0.001) as was the concentration of CD3+ cells (median, 74.8 $\times 10^9$/L vs. 64.3 $\times 10^9$/L; p=0.05).The total number of nucleated cells (TNC; p=0.04) and number of CD34+ cells (p=0.03) per kilogram recipient bodyweight were significantly higher in grafts with inferior quality compared to those with better quality.

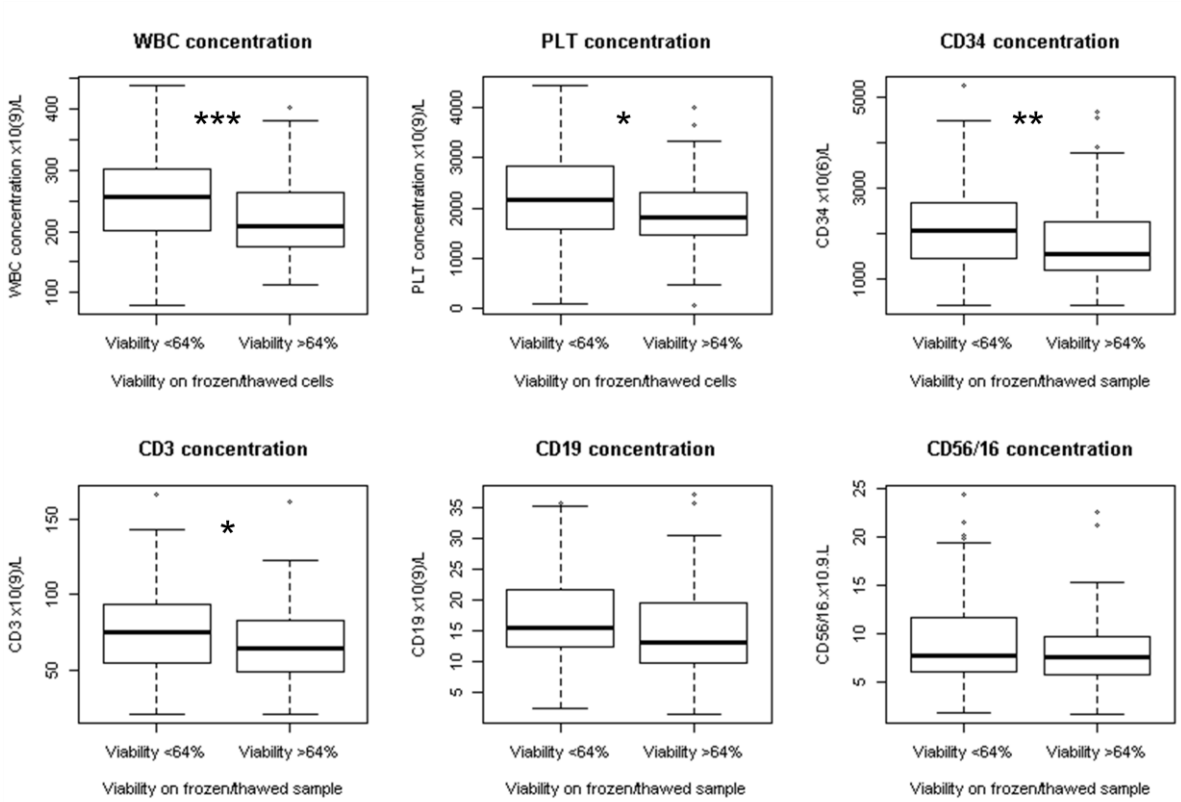

Figure 12: Cellular contents and subsets in grafts with inferior quality (viability <64% in the frozen-thawed vial) and better quality (viability > 64%). *p=0.05, **p=0.01, ***p < 0.001.

Factors influencing graft qualities during liquid storage and transportation have been studied previously (112-117, 134, 261, 266). The picture is complex and multi-factorial. Temperature and duration of storage and transport are known to affect recovery of CD34+ stem cells. The optimum temperature for PBSC storage and transport is 2-8 °C according to Antonenas *et al* (112) and Jansen *et al* (113). Additionally, Jansen and colleagues showed that the WBC concentration affects graft CD34+ cell viability in a time and temperature-dependent manner.

In scientific paper III we confirmed the influence of time from collection to transplantation on graft quality. The median viability in grafts infused the day after collection was 67% compared to 60% for graft transplanted 2 days after collection (p=0.004). The time from

collection to transplant can only be influenced to a certain extent since transit is a necessity for unrelated donor HSCT. Hence, other factors influencing graft quality need to be optimized as well.

In our study there was no significant difference in the PBSC graft temperature on arrival between those grafts with low (<64% viability in frozen-thawed sample) and better quality (>64% viability). However, in our study, temperature was a single-moment measurement at the time of arrival and does not reflect the total transit–storage duration. It is well established that storage and transit temperature affects CD34+ cell viability (112, 113, 117). Furthermore, temperature during storage and transit can be a risk factor for graft rejection (118).

Many studies assessing cellular viability are performed using WBC concentrations far below the cell concentrations we see in PBSC of today (112, 113, 117). In our study the WBC concentration ranged from 78-659 $\times 10^9$/L with a mean of 239 $\times 10^9$/L. When requesting PBSCs from unrelated donors at other centers, we specifically request that the cell graft is to be diluted with donor plasma if the WBC count is higher than 300 $\times 10^9$/L.
Applying a WBC cutoff of 300 $\times$ 10^9/L in revealed that grafts with higher WBC concentrations (n=27) had significantly lower viability in frozen-thawed samples than grafts with a WBC concentration of less than 300 $\times 10^9$/L (n=117; median, 54% vs. 66%, $p<0.001$). Since 27 grafts arrive at our center during the study period with WBC concentration >300 $\times 10^9$/L this request is not always met. In light of these findings we have altered our routine to request addition of donor plasma in all PBSC grafts to be transported, reducing the need for cell enumerations at the time of transit. It is also now possible, in some aphaeresis devises, to add plasma to the collection bag during the aphaeresis procedure which is helpful in countries prohibiting the addition of plasma using sterile connectors outside clean room facilities.

The viability analysis

In this study, the median viability of fresh PBSC was 97% (range, 82%-100%) while the median viability on frozen-thawed samples was 64% (range, 24%-96%). No clear correlation could be found between the viability of WBCs using 7AAD/CD45 on freshly arrived PBSC grafts compared to frozen-thawed vials (Figure 13). There was no correlation between the viability of frozen-thawed cells and the percentage of MNCs in the arriving graft, thus excluding loss of granulocytes to explain the differences.

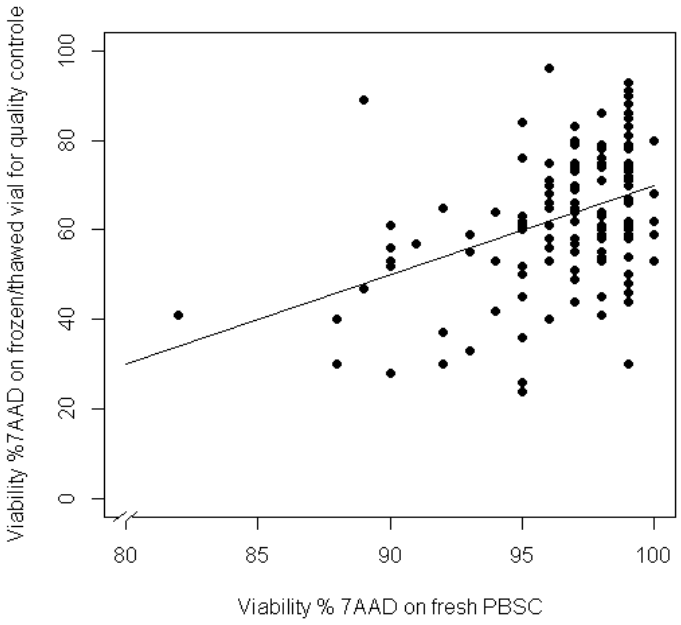

Figure 13: Viability of fresh and frozen/thawed samples. No clear correlation could be found between the viability of WBCs using 7AAD/CD45 on freshly arrived PBSC grafts compared to frozen-thawed vials.

It can be speculated that graft cells in early apoptosis upon time of transport arrival may be pushed into cell death by the freezing-thawing process becoming positive in 7AAD staining. However, this approach relies on a cryopreservation method and the extra processing step may induce additional variation and is thus not suitable as a routine method. The need for additional analysis has been identified by others (134, 266-268) in addition to the in vitro markers used today (CD34+ recovery, cell viability and CFUs). To distinguish early apoptosis, staining for apoptosis using a marker such as annexin V would be useful (267, 269, 270). It has been clearly shown in vitro that the use of, annexin V for example, as a marker for apoptotic cell death is a good complement to markers for cell death.

4.3 GRANULOCYTE TRANSFUSIONS IN ALLOGENEIC HSCT

4.3.1 Scientific paper IV: Granulocyte transfusion against mucositis and infectious complications after allogeneic hematopoietic stem cell transplantation

In total, 421 granulocyte transfusions (GCX) were given to 85 patients, with a median of 4 (range 1-33) granulocyte units transfused per patient. The median number of days between HSCT and GCX was 9 (range 0-240). Most transfusions were given daily. Forty-one (48%) patients received GCX from donors stimulated with steroids only (S-GCX) while 44 (52%) received GCX from donors pre-treated with steroids and G-CSF (GCSF-GCX).

The patient groups (S-GCX/GCSF-GCX and mucositis/infection) did not differ significantly with respect to patient characteristics.

The cell doses were on average 5 fold higher in GCSF-GCX products than in S-GCX products (median 53×10^9 vs. 10×10^9 leukocytes, respectively, $p < 0.001$). The increment of leukocyte and absolute neutrophil counts (ANC) were significantly larger in patients treated with GCSF-GCX products, Figure 14.

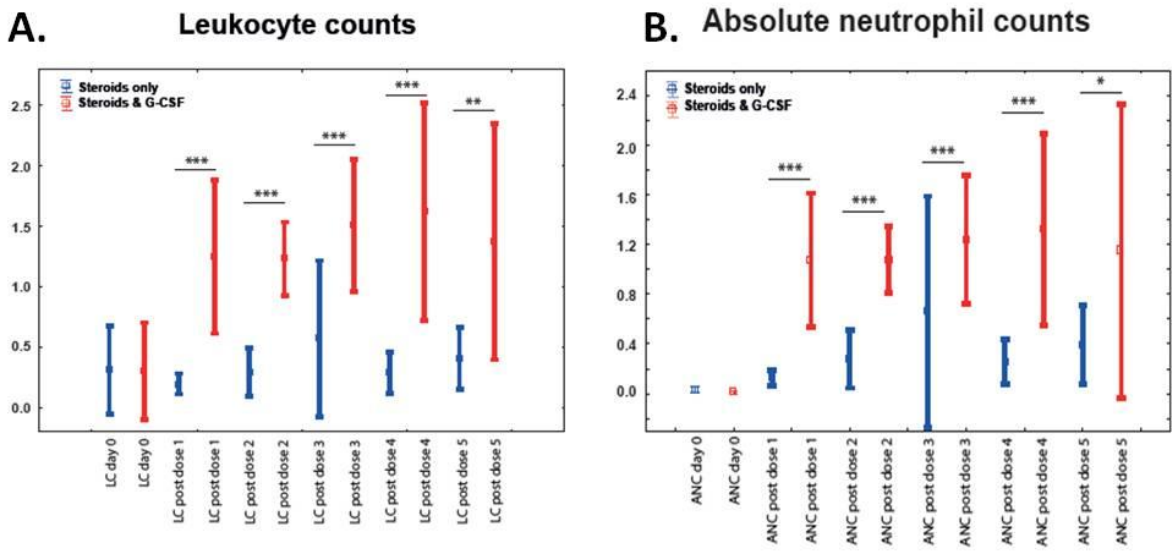

Figure 14: Leukocyte counts (A) and absolute neutrophil counts (B) in peripheral blood of GCX-treated patients. Whiskers indicate 95% confidence interval (red: GCSF-GCX ; blue: S-GCX). *p<0.05, **p≤0.01, ***p < 0.001

The overall response rate (including both partial and complete responses) were similar in patients treated with either GCSF-GCX or S-GCX (83% and 82%, respectively) but there were significantly more complete responses in the GCSF-GCX group compared to the S-GCX group (63 vs. 30%, $p < 0.01$), Figure 15.

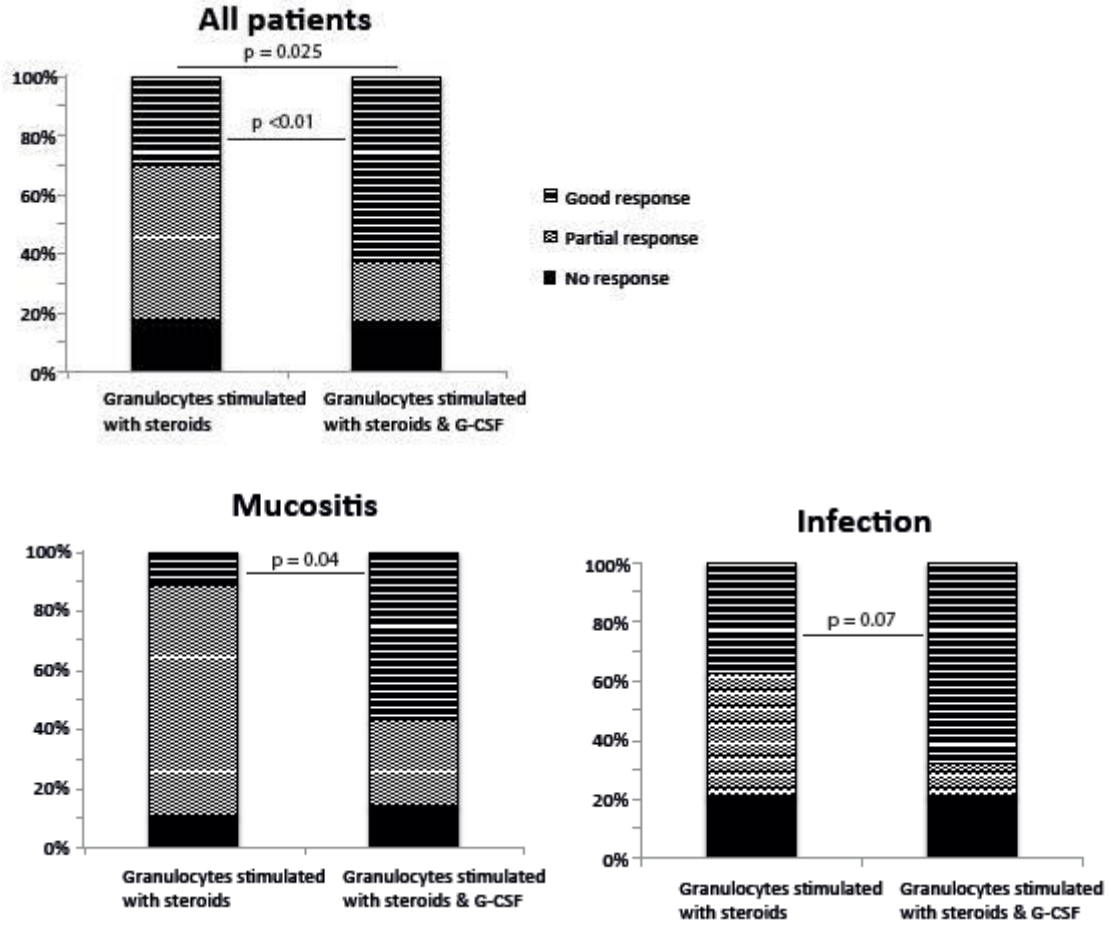

Figure 15: Response to granulocyte transfusion. The percentage of complete, partial and no responses to treatment for all patients, for patients treated due to mucositis and for patients treated due to infection.

In patients with mucositis a similar overall response rate in the GCSF-GCX and S-GCX-treated patients (86% and 89%, respectively) was observed. However, the rate of complete responses was fivefold higher in the GCSF-GCX group (57 vs. 11%, p = 0.04). A trend towards more complete responses was also observed when analyzing patients treated for infection (68 vs. 38%, respectively, p = 0.07). Of the three patients with local bacterial infections treated with S-GCX, two had a partial response and one showed a complete response. All six patients receiving GCSF-GCX for local bacterial infections showed complete treatment responses.

It is debated whether the increment of neutrophils in peripheral blood or clinical outcome, i.e. survival, infection control and adverse events, is correlated to the number of granulocytes in the product. Some studies have shown correlation between dose, increment and effect (197, 198). The tissue infiltration of transfused granulocytes at the site of infection may precede a rise in peripheral blood count and therefore the effect of GCX may not be reflected by increment (191, 199) . A recommended dose of granulocytes at 3×10^8/kg has been suggested (199). In the Council of Europe´s current (2015) guidelines the recommendation is

1.5-3.0 $\times 10^8$ /kg (271). In the UK a granulocyte aphaeresis product should contain $>1 \times 10^{10}$ granulocytes/unit and for whole blood derived granulocytes from 10 buffy coats $>5 \times 10^9$ granulocytes/ unit (adults: 1-2 units /day, children 10-20 mL/kg) (Red Book chapt. 7: www.transfusionguidelines.org). This transfusion dose have also been confirmed in the Cochrane review from 2015 by Estcourt *et al* (194) looking at prophylactic GCX and in the Cochrane report from 2005 by Stanworth *et al* (193) reviewing GCX for treatment of infections.

In our study the median granulocyte transfusion dose of was 5.3×10^{10} for GCSF-GCX and 1×10^{10} for S-GCX. Based on the standards mentioned above, the doses in many of the S-GCX patients were sub-optimal. When looking at response to treatment, the number of non-responders was the same in S-GCX and GCSF-GCX groups. However, the number of complete responders was higher in the GCSF-GCX group and this may be a result of the differences in cell dose.

Adverse events (AE) associated with GCX treatments were experienced by 36 patients (9% of granulocyte transfusions). More AE were reported in patients receiving GCSF-GCX (n=18, 14%) than those receiving S-GCX (n=18, 6%; p=0.02), see Table 7. Twenty-seven were mild to moderate reactions, defined as grade 1-2, with symptoms such as fever, chills, head ache, chest pain, rash or emesis. All symptoms in grade 1-2 AEs were transient and well manageable with symptomatic treatment. Three patients experienced grade 3 AE, one paroxysmal supra-ventricular tachycardia and two patients developed respiratory distress. Six patients developed grade 4-5, AEs with life threatening or lethal respiratory distress requiring oxygen treatment and, in some cases, acute intubation. All the severe pulmonary reactions were seen in patients treated with GCX due to severe systemic infection. Patients with local (non-pulmonary) infections had no reported severe AE.

Table 7: Adverse Events	**All patients n=85**	**S-GCX n=41**	**GCSF-GCX n=44**	
Total granulocyte units transfused	**n=421**	**n=287**	**n=130**	
All adverse events	36 (9%)	18 (6%)	18 (14%)	p=0.02
Severe adverse events; Grade 4-5	6	3 (1.0%)	3 (2.3%)	Ns

Table 7: Reported adverse events (AE) in patients receiving granulocyte transfusions from donors stimulated with steroids only (S-GCX) or steroids and G-CSF (GCSF-GCX). Patients receiving GCSF-GCX had significantly more AE (p=0.02). The frequency of reported severe AE (grade 4-5) did not differ between the groups.

Only one out of six patients with severe pulmonary AEs did not have respiratory symptoms prior to GCX. Therefore we investigated the circumstances of previous pulmonary symptoms and pulmonary AEs, with regard to findings on pulmonary radiologic examinations before and after GCX in the entire patient cohort as seen in Figure 16.

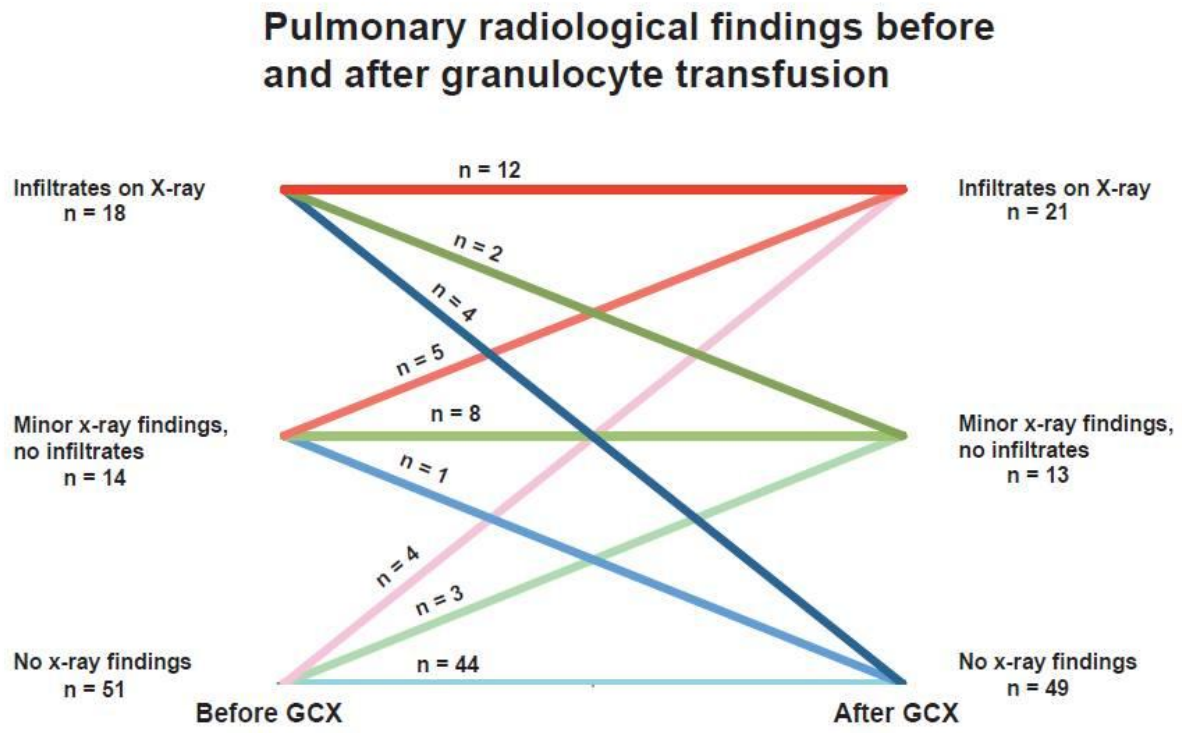

Figure 16: Pulmonary findings in patients prior to and after GCX treatment.

The majority of patients did not experience clinical pulmonary symptoms and had no adverse pulmonary x-ray findings. Six out of 18 patients with pulmonary infiltrates prior to GCX improved their radiological lung status. Four of these six patients had fully resolved pathological findings. Twelve of the patients had remaining pulmonary infiltrates after GCX treatment. Fifty-one patients did not have any pulmonary x-ray findings prior to GCX. Seven of these developed such during treatment, three had minor findings and four had infiltrates.

Pre-existing lung infiltrates have been linked to pulmonary AEs in GCX recipients (208). As a consequence, pulmonary infiltrates are a relative contra-indication to GCX at our center. In this study, a majority of patients that developed life-threatening or fatal pulmonary AEs had symptoms and pathological radiology findings prior to granulocyte transfusion. However, there were also patients that had lung infiltrates prior to GCX whose pulmonary symptoms improved after the treatment and one patient that developed a fatal pulmonary AE who lacked prior pulmonary infiltrates. This indicates that even though a pre-existing pulmonary inflammation is a risk factor for pulmonary AEs, this correlation is not absolute. Pulmonary infiltrates will remain as a relative contra-indication for GCX treatment at our center.

The finding that severe pulmonary AEs were only seen in patients with an underlying systemic infection suggests that a previously existing inflammatory response predisposes to

severe pulmonary AEs after transfusion, as has been previously described for transfusion-related lung injury (TRALI) (272, 273). The clinical presentation of pulmonary AEs in GCX treatment are similar to that in TRALI (273). In TRALI, a two-hit mechanistic hypothesis has been proposed. The "first hit" is constituted by an ongoing inflammatory response in the patient and the "second hit" is effected by the blood product in different manners (272, 273). Supporting evidence for the two hit hypothesis has been published, such as a pre-transfusion increase of serum cytokine in patients who developed TRALI (272). We speculate that similar mechanisms are involved in the pulmonary AEs after granulocyte transfusion. The first hit would be formed by a systemic or pulmonary inflammatory state in the patient and the second hit would depend on the effector functions of the transfused granulocytes. An inflammatory response in the lung tissue would recruit the transfused granulocytes while a systemic inflammation could induce activation of the transfused neutrophils in the blood stream, causing them to adhere and extravasate in the first capillary bed encountered (i.e. the lungs after central venous infusion). This may partly explain why only patients with systemic infection were affected by severe pulmonary AEs in this cohort.

Next we analyzed the impact of granulocyte transfusions on clinical outcome.

The overall one year survival (OS) was 47% in the entire patient cohort. OS was significantly better for patients treated with GCSF-GCX than those treated with S-GCX (59% vs. 34%, $p<0.01$), Table 8 and Figure 17. OS was better for patients treated for mucositis than those treated for infection (62% vs- 40%. $p = 0.02$). There was no difference in one-year OS between patients treated with either GCSF-GCX or S-GCX for infection but in patients treated for mucositis there was a trend towards a higher OS in those receiving GCSF-GCX ($p= 0.08$). There was no significant difference in OS between patients treated with GCX due to mucositis and the matched controls receiving conventional mucositis treatment.

Table 8	**All patients** **n=85**	**S-GCX** **n=41**	**GCSF-GCX** **n=44**	
acute GVHD grade II-IV	38 (45%)	20 (49%)	18 (41%)	Ns
Acute GVHD grade III-IV	12 (15%)	8 (20%)	4 (9%)	Ns
Rejection or graft failure	7 (8%)	3 (7%)	4 (9%)	Ns
Overall survival (OS)	47%	34%	59%	p<0.001

Table 8: Clinical outcome in patients receiving granulocyte transfusions from donors stimulated with steroids only (S-GCX) or steroids and G-CSF (GCSF-GCX). Patients who received GCSF-GCX had significantly better overall survival (OS).

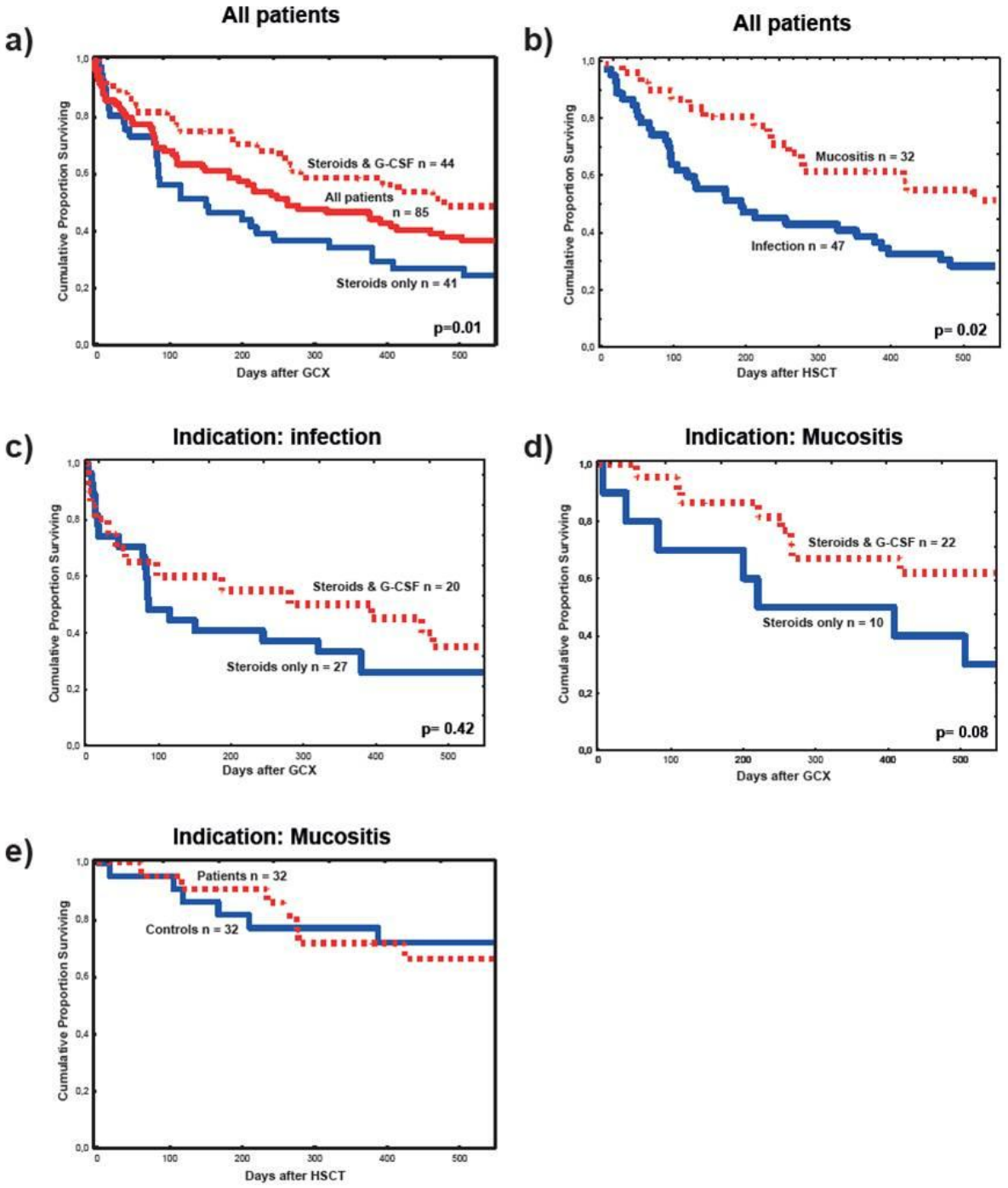

Figure 17: Overall survival (OS) up to 550 days after GCX treatment. S-GCX is displayed as "Steroids only" while the "GCSF-GCX" group as "Steroids & G-CSF". Figure e) displays mucositis patients ("patients") who have received GCX treatment and matched control patients ("controls") who have not received GCX.

The significantly better OS in patients treated for mucositis compared to patients treated for infection is expected, as patients with treatment-refractory infection have a poor prognosis, while severe mucositis has only a moderately increased mortality risk (274, 275).

Patients treated with GCSF-GCX had a higher OS compared to patients treated with S-GCX. This is encouraging and might be partly due to the increased rate of complete responses when using GCSF-GCX compared to S-GCX. However, patients that received GCSF-GCX were all treated after 2005 when G-CSF was added to the granulocyte donor pre-treatment protocol. OS in HSCT patients has increased over time at our center (39) and, since this is a retrospective analysis that included patients from 1998 to 2014, changes in supportive care, treatments and infectious prophylaxis might constitute confounding factors.

The cumulative percentage of acute GVHD grades II-IV has been 30-37% in all patients who underwent allogeneic HSCT at our center during 2001-2009 (39). We did not see any increased rate of acute GVHD grades II-IV in patients receiving GCX treatment (p=0.23). The rate of rejection (9%) in this study was slightly higher but not significantly increased (p=0,9), than what is usually seen at our center (5-6% in the last decade; (241)). There was no significant difference in acute GVHD or rejection/graft failure between patients receiving S-GCX or GCSF-GCX, Table 8.

We studied the effects of GCX treatment using granulocytes from donors pretreated with steroids only (S-GCX) or with steroids and G-CSF (GCSF-GCX) in patients with mucositis or infection. We found no evidence that granulocyte transfusions negatively impact clinical outcome (OS, acute GVHD or rejection/GF). The overall response rate to GCX was similar between these groups but there were more complete responders in the GCSF-GCX group.

Patients with mucositis seemed to benefit more from GCX treatment, especially from GCSF-GCX, and although the total numbers of AEs were not significantly different to that in patients receiving GCX for infection, there were no reported severe AEs in the mucositis group. In patients with severe infection the picture was not as clear, although some patients did respond to treatment. In addition, the risk for severe pulmonary AE, in patients with infections complicated the decision to use granulocytes to treate these patients.

In a Cochrane review by Stanworth *et al* (193) from 2005 (reprinted in 2010), 8 randomized controlled trails including 310 patients with neutropenia treated with GCX for infection were analyzed. The reviewers concluded that the evidence for GCX treatment in these patients was inconclusive.

Recently, results from a multicenter randomized controlled trail (RING study) were published by Price TH *et al* (209). In this study, there was no overall effect of granulocyte transfusion on the primary outcome (survival after 42 days and infection clearance). However, the enrollment in this study only reached half of what was planned, thus, as the investigators concluded, the study lacks sufficient power to detect clinical effect. In a post-hoc analysis

they investigated the role of cell dose and found that patients receiving >6 $x10^8$ neutrophils /kg did significantly better than patients receiving lower doses (<0.6 $x10^8$/kg) or controls.

Thus, the data supporting beneficial effect of GCX in patients suffering from severe infection is still inconclusive. In our study, GCX had no significant detrimental effect on clinical outcomes (OS, GVHD and GF/rejection). However, severe AEs were only found in patients with severe infection which has to be considered when deciding to use GCX in these patients.

5 CONCLUDING REMARKS AND FUTURE PROSPECTS

Many factors influence the outcome after allogeneic stem cell transplantation. The main objective of this thesis was to reach better understanding of the impact of blood group differences, transfusion related complications and graft quality on clinical outcome. This thesis work contributes with pieces to the gigantic HSCT "gig saw puzzle" of answers, questions, data, confounding- and interacting factors.

Scientific paper I & II:

In scientific paper I we found that in leukemia patients undergoing allogeneic HSCT with full myeloablative conditioning there is an increased risk for graft failure in major ABO mismatched donor-recipient pairs, especially in combination with HLA-mismatches.

In scientific paper II we found no impact of ABO donor-recipient mismatch in patients receiving reduced intensity conditioning. Other factors of greater impact may override the effect of ABO donor-recipient differences thus obfuscating its influence and complicating analysis and interpretation of results.

Patients undergoing ABO-non identical HSCT require more red blood cell and platelet transfusions after HSCT.

The presence of ABO antibodies, of donor- or recipient type, not normally found post-HSCT was associated with inferior survival and higher incidence of transplant related mortality. The immunological mechanisms behind this are not fully known, why further studies are needed. The ABO mismatch in itself did not affect clinical outcome, thus the ABO mismatch do not explain this finding.

Scientific paper III:

The data from scientific paper III shows that inferior graft quality, measured as viability on frozen-thawed samples from PBSC grafts, was associated with increased incidence of acute GVHD and TRM. Our study suggests that the clinical follow-up after cell collection and processing as measurement of graft quality needs to be more elaborate and include more variables such as GVHD and TRM. To solely rely on engraftment and survival data is not sufficient as clinical variables for assessment of graft quality.

Further studies are needed to optimize conditions for graft storage and handling. There is also a need for better analyses to assess graft quality in routine transplantation care and for use in validation of cell handling and storage conditions.

At our center Annexin V has been set up for evaluation of apoptosis in grafts. We plan to do follow-up of these data. Preliminary results look promising and were presented at ISBT in London 2015 as an abstract/poster (276).

Scientific paper IV:

In scientific paper IV we could show that neutropenic patients with mucositis seemed to benefit from granulocyte transfusions. For neutropenic patients with infections the effects of treatment were not as clear. In addition, the risk for severe pulmonary adverse events complicates the decision to use GCX treatment in patients with systemic infections. Further studies of GCX treatment of patients with severe infections during neutropenia are needed. The granulocyte cell dose needed to achieve effect of GCX treatment is still not fully elucidated and needs further investigation.

We plan to test a different production method using buffy coat derived granulocytes from whole blood donations (195, 196, 277). By this we hope to achieve a faster delivery of granulocyte products to severely ill patients and sparing G-CSF and steroid treatment of healthy community donors. The two main issues, and thus the study endpoints, with these buffy coat derived granulocyte products are cell dose (and yield) and granulocyte function. We plan to start pilot production this autumn to establish protocol.

6 POPULÄRVETENSKAPLIG SAMMANFATTNING PÅ SVENSKA

Allogen blodstamcellstransplantation är en livräddande behandling för patienter med maligna blodsjukdomar till exempel akuta och kroniska leukemier, medfödda ämnesomsättningssjukdomar eller ärftliga immundefekter.

När en patient behöver transplanteras söker man efter en lämplig donator av blodstamceller. Donatorn behöver ha samma vävnadstyp som patienten, vara HLA-lik. En tredjedel av alla patienter har en HLA-lik, lämplig donator i familjen, vanligen ett syskon. För de resterande två tredjedelarna av patienterna söker man efter en donator i de internationella benmärgsregistren. Att hitta en donator kan vara svårt eftersom det finns en stor variation i HLA mellan individer. Donatorer kan därför finnas långt bort, även på andra kontinenter, vilket ställer stora krav på logistik och transporter.

När en lämplig donator hittats och godkänts efter medicinsk undersökning kan förbehandlingen av patienten med cytostatika och/eller strålning påbörjas. Förbehandlingen kallas konditionering och syftar till att slå ut eventuellt kvarvarande cancerceller samt slå ut patientens egen benmärg och därmed bereda plats åt den nya benmärgen. Konventionell konditionering slår helt ut patientens benmärg helt men det finns även mindre hårda konditioneringsprotokoll, reducerad konditionering, som ibland lämnar kvar en del av patientens egen benmärg.

Blodstamceller, eller graft, kan doneras på olika sätt: benmärg, perifera blodstamceller eller navelsträngsblod. Benmärg skördas med sprutor ur donatorns höftbenskammar under narkos. Vid skörd av perifera blodstamceller ges ett tillväxthormon till donatorn i fem dagar vilket får blodstamceller att vandra från benmärgen ut i blodet. Blodstamcellerna i blodet kan sedan skördas med så kallad aferesteknik. Navelsträngsblod från navelsträng och moderkaka doneras av frivilliga mammor efter förlossningen och förvaras fryst i navelsträngsblodbanker.

Vilket konditioneringsprotokoll och vilken graft sort man väljer beror på patientens sjukdom och tillstånd.

När patienten genomgått sin konditionering skördas blodstamcellerna (graftet) på den plats där donatorn är och transporteras till patientens sjukhus. Graftets innehåll och kvalitet analyseras och ibland behöver graftet behandlas för att minska risken för att patienten ska få en negativ reaktion. Därefter ges graftet till patienten som en transfusion. Patienten har efter konditioneringen nästan inga vita blodkroppar i blodet och är extremt känslig för infektioner. Det tar mellan två och fyra veckor från att graftet transfunderats till att de donerade blodstamcellerna börjat bilda nya blodkroppar.

Transplantation med allogena blodstamceller kan utföras även om patienten och donatorn har olika blodgrupper, exempelvis om patienten är blodgrupp A och donatorn O. Hur blodgruppsskillnader påverkar patienten vid transplantationen är dock inte helt klarlagt.

I den här avhandlingen har vi studerat hur blodgruppsskillnader mellan patient och donator samt antikroppar mot röda blodkroppar påverkar transplantationen. Vidare har vi studerat hur kvaliteten på graftet påverkar patienten. I det sista arbetet har vi studerat hur det går för patienter som fått transfusioner med vita blodkroppar (granulocyter) efter transplantationen på grund av svåra infektioner och svår mucosit (slemhinneinflammation i munnen).

I **arbete I**, som är en retrospektiv (tillbakablickande) studie, ingick 224 patienter som behandlats med transplantation av allogena blodstamceller på grund av leukemi mellan 1997 och 2003. Vi tittade på hur skillnader i blodgrupp mellan donator och patient påverkade risken för så kallad graft failure, det vill säga att graftet inte fungerar i patienten efter transplantationen. Sex av dessa patienter hade graft failure. Vi fann att risken för graft failure var högre hos patienter med olika blodgrupper och hos patienter med HLA-missmatcher (olikheter i HLA).

Arbete II är också en retrospektiv studie men här studerade vi 310 patienter som förbehandlades med så kallad reduced intensity conditioning mellan åren 1998 och 2011 med avseende på hur blodgruppsskillnader och antikroppar mot röda blodkroppar påverkat transplantationen. I den här studien kunde vi inte påvisa att blodgruppsskillnader påverkande transplantationen. Patienter med annan blodgrupp än donatorn behövde dock fler blodtransfusioner än de med lika blodgrupp. Vidare såg vi att patienter som drabbades av blodgruppsspecifika antikroppskomplikationer hade sämre överlevnad.

Resultaten av dessa två studier visar på svårigheten i bedömningen av vilken roll blodgruppsskillnader spelar vid transplantation med blodstamceller. Sannolikt döljer andra, mer betydelsefulla, faktorer en eventuell effekt av skillnader i blodgrupper.

I **arbete III** har vi studerat hur kvaliteten på graftet (blodstamcellerna) påverkar patienten. Vi har mätt graftkvalitet genom att analysera viabilitet på ett fryst/tinat prov från graftet. Viabilitet mäter procenthalten levande celler i ett prov. Att mäta viabilitet direkt på graftet är inte tillräkligt noggrant, om man fryser och tinar provet innan analys så blir metoden känsligare. Det visade sig att gruppen patienter som transplanterades med graft av sämre kvalitet (det vill säga hade lägre viabilitet) fick mer komplikationer med akut transplantat mot värd reaktion (graft versus host disaese, GVHD) och avled i högre grad i så kallad transplantationsrelaterad mortalitet. Vidare noterade vi att en bidragande orsak till dålig graftkvalitet var höga cellkoncentrationer.

Denna studie visade att graftkvalitet påverkar hur det går för patienten efter transplantationen. Det är därför av stor vikt att förvaring och transport av graft sker under optimala förhållanden.

Arbete IV. Vissa patienter som transplanterats med blodstamceller kan under första tiden efter transplantationen drabbas av svåra infektioner och svår så kallad mucosit (slemhinneinflammation i munnen). Dessa patienter kan under en tid efter transplantationen behöva transfunderas med vita blodkroppar (granulocyter) från friska blodgivare. Vid granulocytgivning förbehandlas blodgivaren antingen med kortison enbart eller med kortison och tillväxthormon i kombination. I denna studie ingick 85 patienter. Patienterna svarade totalt sett på behandling i lika hög grad oavsett vilken förbehandling blodgivaren fått. Dock var det fler som blev helt återställda av de patienter som fått granulocyter där blodgivaren förbehandlats med en kombination av kortison och tillväxthormon. Patienter som behandlats med granulocyttransfusioner på grund av mucosit hade god effekt av behandlingen. För patienter som behandlades på grund av svår infektion är bilden inte lika tydlig, vissa har effekt av granulocyttransfusioner och andra inte. Totalt erfor 36 patienter någon biverkan vid en granulocyttransfusion. Det var ingen skillnad i biverkningsfrekvens beroende på vilken förbehandling blodgivaren fått. Av dessa 36 biverkningar var 6 allvarliga lungbiverkningar. Samtliga svåra biverkningar var hos patienter som behandlats på grund av svår infektion vilket ytterligare försvårar bedömningen om granulocyttransfusioner bör ges till en patient med svår infektion.

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